RESEARCH NOTES

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Seroprevalence of *Toxoplasma gondii* Antibodies in Cats From Durango City, Mexico

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ABSTRACT: The prevalence of antibodies to *Toxoplasma gondii* was determined in sera from 105 domestic cats from Durango City, Mexico. Using a modified agglutination test, antibodies to this parasite were found in 21% of the 105 cats, with titers of 1:25 in 3 cats, 1:50 in 4 cats, 1:200 in 5 cats, 1:400 in 2 cats, 1:800 in 2 cats, 1:1,600 in 4 cats, and 1:3,200 or higher in 2 cats. Cats older than 1 yr had a significantly higher frequency of infection than that found in cats younger than 0.5 yr (41 vs. 13.2%, respectively; odds ratio = 4.55; 95% CI = 1.24–17.18; $P = 0.01$). Overall, the seroprevalence of *T. gondii* antibodies in cats in Durango, Mexico, is much lower compared with those reported in other countries.

Cats are essential in the life cycle of *Toxoplasma gondii* because they are the only hosts that can excrete the environmentally resistant oocysts in nature (Dubey and Beattie, 1988). Little is known of the prevalence of *T. gondii* in cats in Mexico. In the present report, we determined the prevalence of *T. gondii* infection in cats from Durango City, Mexico, and we attempted to identify general characteristics of cats associated with infection.

All 105 unwanted cats enrolled from August to November 2006 in the animal shelter in Durango City, Mexico, were studied. The animal shelter receives stray cats captured by the municipality from the streets of Durango City and unwanted pet cats given by the owners for adoption. General data, including age, breed, gender, health status, origin (stray or household), type of food eaten, and residence for cats were obtained (Table I).

The sera were transported by courier from Mexico to Beltsville, Maryland, where serology was performed. Two-fold serial dilutions were made (1:25–1:3,200) and tested with a modified agglutination test (MAT), as described previously (Dubey and Desmonts, 1987). Whole formalin-fixed tachyzoites and mercaptoethanol were used as antigen, and a titer of 1:20 or higher was considered indicative of *T. gondii* exposure based on experimental studies in cats (Dubey and Thulliez, 1989; Dubey et al., 1995a, 1995b).

Results were analyzed with the aid of the software Epi Info 6. For comparison of the frequencies among the groups, the Mantel-Haenszel test, and when indicated the Fisher exact test, were used. The association of the animal characteristics and the *T. gondii* infection was assessed by calculating the odds ratio (OR) with a 95% confidence interval (CI). For age comparison among groups of cats the Student's *t*-test was used. A P value of less than 0.05 was considered significant.

Antibodies to *T. gondii* were found in 21% of the 105 cats, with titers of 1:25 in 3 cats, 1:50 in 4 cats, 1:200 in 5 cats, 1:400 in 2 cats, 1:800 in 2 cats, 1:1,600 in 4 cats, and 1:3,200 or higher in 2 cats.

General characteristics of 105 cats are shown in Table I. All cats were of crossbreed and resided in urban areas. Most cats studied were females, and the frequency of *T. gondii* infection observed in these females was similar to that observed in male cats ($P = 0.56$). Cats were 1 mo to 7 yr old (mean 9 mo), and the prevalence of infection increased with age. Most cats were healthy, and the prevalence of infection in this group did not differ from that in ill cats ($P = 0.33$). In addition, most cats studied were pets. The prevalence of infection in male and female cats in the stray group was comparable (33.3 and 31.3%, respectively; $P = 0.61$). Similarly, although the prevalence of infection in pet female cats (20%) was 2-fold higher than that observed in pet male cats (9.1%), the overall prevalence of infection in male and female cats did not differ significantly ($P = 0.21$).

Cats older than 1 yr had a significantly higher frequency of infection than cats younger than 0.5 yr (41 vs. 13.2%, respectively; OR = 4.55; 95% CI = 1.24–17.18; $P = 0.01$) and slightly higher than that observed in cats 0.5 to 1 yr old (41 vs. 20%; $P = 0.18$). *Toxoplasma gondii* seroprevalence in stray cats is much higher in stray versus pet cats (Dubey, 1973; DeFeo et al., 2002; Nutter et al., 2004) as was the case in the present study. Higher seroprevalence in adult cats versus kittens, observed in the present study, supports earlier findings (Dubey, 1973; Ruiz and Frenkel, 1980b; Pena et al., 2006) and relates to the life cycle of *T. gondii* in cats; most cats are thought to become infected with *T. gondii* after weaning when they begin to hunt for food.

The 21% prevalence of *T. gondii* antibodies in cats of Durango City, Mexico, in the present study is the lowest among all other surveys from North and South America, West Indies, and 1 study from Europe using a cut-off MAT titer of 1:20 (Table II). The prevalence of *T. gondii* in cats is a reflection of prevalence of *T. gondii* in animals that cats access for food. For example, Ruiz and Frenkel (1980a, 1980b) found a very high prevalence of *T. gondii* in cats and rodents and free-range chickens from Costa Rica. Although there are several reports of *T. gondii* infection in humans and animals in Mexico (Varela et al., 1961; Fernandez-Torrano et al., 1986; Velasco-Castrejon et al., 1992; Galvan Ramirez et al., 1995, 1997; Del Rio-Chiriboga et al., 1997; Dubey, Morales, and

TABLE I. General characteristics of the cats and prevalence of *T. gondii* antibodies.

Characteristic	Cats studied		Cats positive	
	No.	%	No.	%
Gender				
Male	34	32.4	6	17.6
Female	71	67.6	16	22.5
Age groups (yr)				
<0.5	53	50.4	7	13.2
0.5–1	30	28.6	6	20
>1	22	21	9	41
Residence area				
Urban	105	100	22	21
Health status				
Healthy	95	90.5	21	22.1
Ill	10	9.5	1	10
Origin				
Stray	28	25.7	9	32.1
Household	77	73.3	13	16.9
Breed				
Crossbreed	105	100	22	21
Food				
Commercial	91	86.7	19	20.9
Homemade	66	62.9	13	19.7
Hunting and garbage	30	28.6	9	30

TABLE II. Seroprevalence* of *T. gondii* antibodies in cats from different countries.

Country	City or region	Source	No. tested	% Prevalence	Reference
Brazil	São Paulo state	Stray, unwanted pets	502	26.3	Silva et al. (2002)
	São Paulo state	Stray, unwanted pets	237	35.4	Pena et al. (2006)
	Paraná state	Stray	58	84.4	Dubey, Navarro et al. (2004)
Colombia	Armenia and Bogotá	Stray, unwanted pets	170	30.5	Dubey, Su et al. (2006)
People's Republic of China	Guangzhou	Market	34	79.4	Dubey et al. (2007)
Spain	Barcelona	Pets	220	45	Gauss et al. (2003)
United States	Ohio	Pets and stray	275	48	Dubey et al. (2002)
	Rhode Island	Pets and stray	200	42	DeFeo et al. (2002)
	Illinois	Farms	295	75.6	Dubey, Weigel et al. (1995)
	North Carolina	Stray and pets	176	50.5	Nutter et al. (2004)
West Indies	Grenada	Pets	40	35	Asthana et al. (2006)
	St. Kitts	Pets	106	84.9	Moura et al. (2007)

* Using modified agglutination test titer of 1:20 or higher.

Lehmann, 2004; Figueroa-Castillo et al., 2006), little is known of the epidemiology of this parasite in Mexico. Varela et al. (1961) found dye test antibodies in 52.2% of cats in Mexico, but they provided no other details about the cats. Galvan Ramirez et al. (1999) reported that 64% of 59 cat owners and 70.8% of their cats in Guadalajara had *T. gondii* antibodies. *Toxoplasma gondii* oocysts were found in the feces of 13 of 200 cats from Mexico (de Aluja and Aguilar, 1977). For epidemiologic studies, serologic surveys in cats are more useful than the detection of oocysts, because at any given time only 1% of cats shed oocysts in their feces (Dubey, 2004). In the present study, 21% of cats were seropositive for *T. gondii*, and they likely were shedding oocysts, thereby contaminating the environment.

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Prevalence of *Neospora caninum* and *Toxoplasma gondii* Antibodies in Wild Ruminants From the Countryside or Captivity in the Czech Republic

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ABSTRACT: In the Czech Republic, sera from 720 wild ruminants were examined for antibodies to *Neospora caninum* by screening competitive-inhibition enzyme-linked immunosorbent assay and confirmed by indirect fluorescence antibody test (IFAT); the same sera were also examined for antibodies to *Toxoplasma gondii* by IFAT. *Neospora caninum* antibodies were found in 14% (11 positive/79 tested) roe deer (*Capreolus capreolus*), 14% (2/14) sika deer (*Cervus nippon*), 6% (24/377) red deer (*Cervus elaphus*), 1% (2/143) fallow deer (*Dama dama*), 3% (3/105) mouflon (*Ovis musimon*), and none of 2 reindeer (*Rangifer tarandus*). *Toxoplasma gondii* antibodies were found in 50% (7/14) sika deer, 45% (169/377) red deer, 24% (19/79) roe deer, 17% (24/143) fallow deer, 9% (9/105) mouflon, and 1 of 2 reindeer. In 42 samples of wild ruminants that tested positive for *N. caninum* antibodies, 28 (67% of the positive *N. caninum* samples) reacted solely to *N. caninum*. This is the first evidence of *N. caninum* infection in mouflon, the first *N. caninum* seroprevalence study in farmed red deer, and the first survey of *N. caninum* in wild ruminants from the Czech Republic.

Toxoplasma gondii and *Neospora caninum* are 2 closely related protozoan parasites that are distributed worldwide. Both species have an indirect life cycle, with carnivores as the definitive hosts (Dubey and Beattie, 1988; Dubey, 2003). Definitive hosts of *N. caninum* are dogs and coyotes (*Canis latrans*) that excrete oocysts in feces (Dubey, 2003; Gondim, 2004). Wild herbivores are suggested to play a role of intermediate hosts in the sylvatic cycle of *N. caninum* infection (Gondim, 2006). In North America, antibodies against *N. caninum* have been found in white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*), caribou (*Rangifer tarandus*), bison (*Bison bison*), and muskox (*Ovibos moschatus*) (Dubey et al., 1999; Lindsay et al., 2002; Gondim et al., 2004; Dubey and Thulliez, 2005). In Europe, antibodies against *N. caninum* were detected in red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), chamois (*Rupicapra rupicapra*), Alpine ibex (*Capra ibex*), Barbary sheep (*Ammotragus lervia*), and in European bison (*Bison bonasus*; Ferroglio and Rossi, 2001; Ferroglio et al., 2001; Cabaj et al., 2005; Almeria et al., 2006).

Definitive hosts of *T. gondii* are cats and other felids (Dubey and Beattie, 1988). Many species of warm-blooded animals, including humans, serve as intermediate hosts; hosts can be infected by ingestion of food or water contaminated with sporulated *T. gondii* oocysts excreted by the definitive host, by consumption of cysts in infected animals tissues, or congenitally (Dubey and Beattie, 1988). As herbivorous animals, the wild ruminants are a good indicator for the monitoring of environmental contamination with *T. gondii* oocysts. Meat or venison of wild ruminants infected with *T. gondii* could serve as a potential source of toxoplasmosis for other animals, especially carnivores, including humans. Toxoplasmosis infection was documented in deer hunters from the United States after ingesting undercooked or raw deer venison (Sacks et al., 1983; Ross et al., 2001). In North America, antibodies against *T. gondii* have been found in white-tailed deer, black-tailed deer (*Odocoileus hemionus*), marsh deer (*Blastocerus dichotomus*), pampas deer (*Ozotocerus bezoarticus*), moose, caribou, bison, Dall sheep (*Ovis dalli*), and muskox (Lindsay et al., 1991; Chomel et al., 1994; Ferreira et al., 1997; Kutz et al., 2000, 2001; Zarnke et al., 2000). In Europe, antibodies to *T. gondii* have been reported in red deer, roe deer, fallow deer (*Dama dama*), reindeer (*Rangifer tarandus*), moose, chamois, mouflon (*Ovis musimon*), Spanish ibex (*Capra pyrenaica*), and Barbary sheep (Catar, 1972; Kapperud, 1978; Williamson and Williams, 1980; Hejlicek et al., 1997; Oksanen et al., 1997; Sroka, 2001; Vikoren et al., 2004; Gauss et al., 2006).

The aim of this study was to survey the seroprevalence *N. caninum* and *T. gondii* antibodies in wild ruminants from the countryside and captivity in the Czech Republic. During 1998–2006, sera from 720 wild ruminants from 11 of 14 existing Czech regions (Prague, Stredocesky, Ustecky, Karlovarsky, Plzensky, Jihocesky, Liberecky, Vysocina, Moravskoslezsky, Olomoucky, and Jihomoravsk) were collected.

Wild ruminants came from game preserves (a fenced hunting district with more than 50 ha, where animals are bred mostly for hunting purposes but can, in some cases, be released into the wild), farms (fenced area, where the size is not limited, but usually is smaller than game preserves; animals are bred as farm animals), or free range.

Blood samples were taken from 377 red deer, primarily from 4 farms and 4 game preserves (but in 1 case, a free-ranging animal), 79 free-ranging roe deer, and in 10 cases, from 2 game preserves; 14 sika deer (*Cervus nippon*) from 2 game preserves; 143 fallow deer from 6 game preserves and 79 free-ranging cases; 76 free-ranging mouflons and 29 from 2 game preserves; and 2 reindeer from 1 farm. The blood samples were obtained from blood vessels of animals before transportation or from hearts of animals that were shot during hunting seasons.

Blood was centrifuged and sera were stored at −20 C until assayed for antibodies to *N. caninum* and *T. gondii*. A commercial competitive-

TABLE I. Prevalence of *N. caninum* (cELISA, IFAT) and *T. gondii* (IFAT) in wild ruminants from The Czech Republic.

	Neospora caninum					*Toxoplasma gondii*				IFAT-positive for both *N. caninum* and *T. gondii*
Species	Examined	Positive (cELISA)	Positive (IFAT)*	Prevalence (%)†	Inhibition range (cELISA)	Examined	Positive (IFAT)	Prevalence (%)	Antibody titer range	
Red deer (*Cervus elaphus*)	377	24	24	6	30.4–85	377	169	45	40–640	18/377 (5%)
Sika (*Cervus nippon*)	14	2	2	14	34–56	14	7	50	80–320	0/14 (0%)
Fallow deer (*Dama dama*)	143	6	2	1	30.9–39	143	24	17	40–160	2/143 (1%)
Roe deer (*Capreolus capreolus*)	79	12	11	14	59–91	79	19	24	40–160	7/79 (9%)
Mouflon (*Ovis musimon*)	105	4	3	3	45–56	105	9	9	40–320	1/105 (1%)
Reindeer (*Rangifer tarandus*)	2	0	0	0	0	2	1	50	80	0/2 (0%)
Total	720	48	42	6	30.4–91	720	229	32	40–640	14/720 (4%)

* For confirming, the samples positive in screening cELISA were examined by IFAT.
† Prevalence was counted from samples that were positive in both of the tests.

inhibition enzyme-linked immunosorbent assay (cELISA; VMRD, Pullman, Washington) was used for detection of *N. caninum* antibodies in wild ruminants according to the manufacturers' instructions. The sera were positive if more than 30% inhibition was found. To confirm results in cELISA with a more sensitive method, the positive sera were retested by direct fluorescence antibody test (IFAT) with a commercially available *Neospora* NIFR antigen (VMRD), anti-deer IgG, and anti-goat IgG conjugate (VMRD). The sera were diluted in a 2-fold series starting at 1:50 as a basic dilution; a titer ≥1:50 was considered positive. Only animals that were positive to both tests were considered positive.

Toxoplasma gondii antibodies were detected in sera by IFAT with a commercially available antigen Sevatest toxoplasma NIFR (Sevapharma, Prague, The Czech Republic) and anti-deer IgG conjugate (KPL Inc., Gaithersburg, Maryland) and anti-goat IgG conjugate (VMRD). The sera were diluted in a 2-fold series starting at 1:40 as a basic dilution; a titer ≥1:40 was considered positive.

The results of a serologic survey of *N. caninum* and *T. gondii* antibodies (prevalence, inhibition, and titer ranges) in wild ruminants in the Czech Republic are presented in Table I. This report presents the first evidence of *N. caninum* infection in mouflon; Almeria et al. (2006) examined 27 mouflons from Spain, but with negative results. The highest prevalence of *N. caninum* was observed in roe deer and sika deer, followed by red deer, and low prevalence in fallow deer and mouflons. Similar results were reported by Almeria et al. (2006) from Spain. *Neospora caninum* antibodies were found in 12% (28/237) of the red deer and in 6% (2/33) of the roe deer; mouflon and fallow deer were negative. A possible explanation for higher prevalence in roe deer and red deer might be related to their grazing in the lowlands close to human settlements, where conditions are more conductive to *N. caninum* transmission. Wild ruminants probably become infected by ingesting food or water contaminated by *N. caninum* oocysts excreted by canids in the area (Almeria et al., 2006). On the other hand, wild or domestic dogs in rural areas could be infected by eating offal from wild ruminants left in the forest by hunters.

Positive reaction in IFAT for both *N. caninum* and *T. gondii* were observed in 4% of examined animals (Table I). In 42 samples of wild ruminants that tested positive for *N. caninum* antibodies, 28 (67% of the positive *N. caninum* samples) reacted solely to *N. caninum*. Similarly, Almeria et al. (2006) found that 70% of their positive samples reacted solely to *N. caninum*, indicating less cross-reaction between these 2 closely related parasites.

Here, we also present the first evidence of *T. gondii* antibodies in sika deer and reindeer in the Czech Republic. We can compare our results with a previous *T. gondii* seroprevalence study in a group of wild ruminants in the Czech Republic that was done in South Bohemia during 1981–1990 (Hejlicek et al., 1997). Sera of fallow deer, red deer, roe deer, and mouflons were examined by the Sabin–Feldman dye test, with 100% (3/3), 15% (46/303), 14% (13/95), and 10% (2/20) prevalence, respectively. We found a higher prevalence in red deer (45%) and roe deer (24%), but lower in fallow deer (16.8%) and mouflons (8.6%). Red deer from our study came mostly from farms and game preserves, whereas in the previous study (Hejlicek et al., 1997), most of the animals were free ranging. Fenced areas, especially farms, are usually smaller, and cats had greater access to these areas. The main source of *T. gondii* infection for wild ruminants is feedstuff (grass, hay, or commercial feed) or water contaminated with *T. gondii* oocysts passed by felids in their feces (Dubey and Beattie, 1988). Other carnivores are also at risk for *T. gondii* infection, as well as humans after consumption of meat or offal from wild ruminants that are infected by *T. gondii* (Sacks et al., 1983; Ross et al., 2001).

Similar to Almeria et al. (2006), we found that animals from certain farms or game preserves were more infected than animals from other localities. We can conclude that *T. gondii* and *N. caninum* infection are enzootic in character. In our study, the majority of red deer came from 2 large farms (i.e., 1 farm from the Stredocesky region, where 9 and 51% of 141 examined animals had antibodies against *N. caninum* and *T. gondii*, respectively, and another farm in the Plzensky region, where 188 animals were examined, with 4 and 41% *N. caninum* and *T. gondii* prevalence, respectively. All positive samples in sika deer were found in 1 game preserve (animals were previously imported from Germany) in the Vysocina region, with 20 and 70% (of 10 examined animals) *N. caninum* and *T. gondii* prevalence, respectively. All *N. caninum*– and *T. gondii*–positive samples in roe deer were found in animals from 1 game preserve in the Plzensky region, with 33% (1/3) of both *N. caninum* and *T. gondii* prevalence, and from free-ranging animals in the Vysocina region, where 16 and 26% of 69 ruminants had antibodies against *N. caninum* and *T. gondii*, respectively. All *N. caninum*–positive samples and the majority of all *T. gondii*–positive samples from mouflons were found in free-ranging animals from the Ustecky region, where 25% (3/12) and 42% (5/12) had antibodies against *N. caninum* and *T. gondii*, respectively, and from the Stredocesky region, where prevalences were 6% (1/17) for *N. caninum* and 12% (2/17) for *T. gondii*. Finally, half of all *T. gondii*–positive fallow deer came from a single game preserve in the Stredocesky region, where 29% (of 41 examined animals) were positive.

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Survey of the Metazoan Ectoparasites of the European Flounder *Platichthys flesus* (Linnaeus, 1758) along the North-Central Portuguese Coast

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ABSTRACT: A survey was undertaken to identify metazoan ectoparasite species on the European flounder, *Platichthys flesus* (Linnaeus, 1758), in 4 different locations off the north-central Portuguese coast. Parasites of 7 different taxa were found: *Caligus diaphanus*, *Caligus* sp., and *Lepeophtheirus pectoralis* (Copepoda: Caligidae); *Acanthochondria cornuta* (Copepoda: Chondracanthidae); *Holobomolochus confusus* (Copepoda: Bomolochidae); *Nerocila orbignyi* (Isopoda: Cymothoidae); and praniza larvae (Isopoda: Gnathiidae). *Lernaeocera branchialis*, a common European flounder parasite in the North and Baltic Seas, was not observed among the surveyed fish. *Caligus diaphanus*, *Caligus* sp., and *Nerocila orbignyi* are new host records. The high prevalence and intensity values recorded for *L. pectoralis* and *A. cornuta* suggest that both parasite species are common to the European flounder along the north-central Portuguese coast. In contrast, infection levels with respect to the other parasite taxa were, in most cases, comparatively lower, thereby indicating that they only occur occasionally among flounders in the surveyed area.

The European flounder *Platichthys flesus* (Linnaeus, 1758) (Teleostei: Pleuronectidae) is a catadromous flatfish species that spends much of its life cycle in estuarine and brackish aquatic environments, going to the open sea to spawn in early spring. Its geographic distribution extends along the Atlantic coast, from the White Sea in the north, to northern Africa in the south, including also the Mediterranean and the Black seas (Lucas and Baras, 2001). It is an important species to the Portuguese fisheries, occurring along the entire coast of Portugal (Sobral and Gomes, 1997).

Several metazoan ectoparasite species have already been recorded on

TABLE I. Metazoan ectoparasitic species recorded for the European flounder *Platichthys flesus* (Linnaeus, 1758) in different studies of the literature and respective prevalence values (range).

Group: Family Species	Geographic location	Prevalence (%)	Reference
Monogenea: Gyrodactylidae			
Gyrodactylus unicopula Glukhova, 1955	Baltic Sea	0.4–2.0	Chibani and Rokicki, 2004; Chibani et al., 2005
	North Sea	*	MacKenzie and Gibson, 1970
Gyrodactylus flesi Malmberg, 1957	Baltic Sea	0.1–0.5	Chibani and Rokicki, 2004; Chibani et al., 2005
Gyrodactylus sp.	North Sea	1.1	Schmidt, 2003
Copepoda: Caligidae			
Caligus curtus Müller, 1785	Norwegian Sea	*	Lile et al., 1994
Caligus elongatus von Nordmann, 1832	North Sea	3.3–28	Boxshall, 1974; Schmidt, 2003
Lepeophtheirus pectoralis (Müller, 1777)	North Sea	78.4–96	Boxshall, 1974; Schmidt, 2003
	Ythan Estuary	*	MacKenzie and Gibson, 1970
	Thames River	0.5–13.3	El-Darsh and Whitfield, 1999
	Norwegian Sea	*	Lile et al., 1994
	Atlantic Ocean	52.5–79.4	Marques et al., 2006
Lepeophtheirus europaensis (Zeddam, Berrebi, Renaud, Raibaut, and Gabrion, 1988)	Mediterranean Sea	*	Zeddam et al., 1988
Copepoda: Pennellidae			
Lernaeocera branchialis (L.)	Baltic Sea	4–88	Køie, 1999
	North Sea	67–92.6	Boxshall, 1974; Schmidt, 2003
	Ythan Estuary	*	MacKenzie and Gibson, 1970
	Thames River	8.9	El-Darsh and Whitfield, 1999
	Norwegian Sea	*	Lile et al., 1994
Copepoda: Chondracanthidae			
Acanthochondria cornuta (Müller, 1776)	North Sea	50–63.7	Boxshall, 1974; Schmidt, 2003
	Ythan Estuary	*	MacKenzie and Gibson, 1970
	Atlantic Ocean	10.5–76.3	Kabata, 1959; Marques et al., 2006
	Norwegian Sea	*	Lile et al., 1994
Acanthochondria soleae (Krøyer, 1838)	Atlantic Ocean	*	Kabata, 1959
Acanthochondria limandae (Krøyer, 1863)	Atlantic Ocean	*	Kabata, 1959
Copepoda: Bomolochidae			
Holobomolochus confusus (Stock, 1959)	Baltic Sea	32	Køie, 1999
	North Sea	4.7	Schmidt, 2003
Isopoda: Gnathiidae			
Gnathia sp.	Atlantic Ocean	1.3	Marques et al., 2006

* Present.

the European flounder, *P. flesus* (L.), and reported in different studies of the literature (see Table I). However, for south European waters, only a single record indicating a flounder's infection by a new species, *Lepeophtheirus europaensis*, in the Mediterranean Sea (Zeddam et al., 1988), and a survey reporting flounder's infection by 3 different ectoparasite species in the south-central Portuguese coast (Marques et al., 2006), are known. Indeed, as far as we are aware, no parasitological survey has yet been conducted for flounders off the northern Portuguese coast, the geographic area where the economic income from flounder fishing is most important. Moreover, according to Lile et al. (1994), fish parasite communities often vary considerably in composition over short to moderate distances. Therefore, the main aim of the present study was to characterize the flounder's metazoan ectoparasite assemblage along the north-central Portuguese coast from different sampling locations.

On 2 and 8 September 2005, 120 flounders from 4 locations off the north-central Portuguese coast, i.e., Viana do Castelo (VC) (41°40′N, 8°50′W), Matosinhos (M) (41°10′N, 8°42′W), Aveiro (A) (40°38′N, 8°45′W), and Figueira da Foz (FF) (40°8′N, 8°52′W) (Fig. 1), were collected for examination of metazoan ectoparasites. In each location, 30 fish were collected by random sampling from the nets of local fishing boats. All the fish were kept frozen at −20 C until they could be examined. Each specimen was weighed (mean ± SD [minimum–maximum] = 279.2 ± 172.8 [160.7–1,090.4] g [VC]; 314.5 ± 217.6 [139.4–1,124.0] g [M]; 267.4 ± 122.0 [113.6–613.8] g [A]; 409.9 ± 207.3 [158.4–836.2] g [FF]), measured (27.6 ± 3.7 [24.2–42.8] cm [VC]; 28.7 ± 4.9 [23.5–42.7] cm [M]; 27.5 ± 4.3 [19.8–38.6] cm [A]; 30.9 ± 5.0 [23.6–41.6] cm [FF]), and sexed (20 males and 10 females [VC]; 10 males and 20 females [M]; 9 males and 21 females [A]; 11 males and 19 females [FF]). The body skin, eyes, fins, branchial chambers (subopercular surfaces, walls, gill arches, and pseudobranchiae), and nasal and buccal cavities were examined for metazoan ectoparasites using a stereomicroscope. Collected specimens were cleaned and then fixed in 70% alcohol. Later, copepods were cleared in 90% lactic acid (Humes and Gooding, 1964). Parasites were identified according to Naylor (1972) and Bruce (1987) for Isopoda, and to Kabata (1979, 1992) for Copepoda. It was not possible to identify the gnathiid pranizae at the species level because the identification keys require adult male specimens that were not found in our survey. Nevertheless, all the female larvae presented the same morphological type, which is, presumably, an indication of a single species.

After evaluating the sites of parasite infection on the host's body surface, the following ecological parameters were determined according to Bush et al. (1997) for each of the 4 sampled locations: prevalence (number of infected fish/percentage of infected fish [95% confidence

TABLE II. Metazoan ectoparasitic taxa recorded on flounders from the 4 sampled locations off the north-central Portuguese coast, their sites of infection, infection parameters (number of infected fish/prevalence [95% confidence interval]%, mean intensity ± SD [range]), and first-order jackknife estimator of species richness (estimated richness ± SD [$N = 30$ fish for all sampled locations]).

Parasite group			Sampled location			
Family	Taxa	Host site*	Viana do Castelo	Matosinhos	Aveiro	Figueira da Foz
Copepoda						
Caligidae	*Caligus diaphanus*	B	—	—	1/3 (0–17) (1)	—
	Caligus sp.	B; F	—	5/17 (6–35) (1)	—	—
	Lepeophtheirus pectoralis	B; F	6/20 (8–39) 7.2 ± 7.2 (1–19)	30/100 (88–100) 14.1 ± 9.9 (3–53)	29/97 (83–100) 7.6 ± 6.9 (1–34)	28/93 (78–99) 9.5 ± 10.0 (1–50)
Chondracanthidae	*Acanthochondria cornuta*	B; F; SOS; GA; P	5/17 (6–35) 22.0 ± 12.8 (5–41)	30/100 (88–100) 47.6 ± 22.6 (8–96)	29/97 (83–100) 34.4 ± 24.2 (2–110)	30/100 (88–100) 38.1 ± 25.9 (4–104)
Bomolochidae	*Holobomolochus confusus*	NC	—	1/3 (0–17) (1)	—	—
Isopoda						
Cymothoidae	*Nerocila orbignyi*	F; GA	—	3/10 (2–27) (1)	—	—
Gnathiidae	Praniza larvae	B; F; BC; GA	20/67 (47–83) 1.7 ± 0.9 (1–4)	—	1/3 (0–17) (3)	—
	Estimated richness S_{JK}		3 ± 0.0	6 ± 1.0	6 ± 1.3	2 ± 0.0

* B, body; BC, buccal cavity; F, fins; GA, gill arches; NC, nasal cavities; P, pseudobranchiae; SOS, subopercular surfaces.

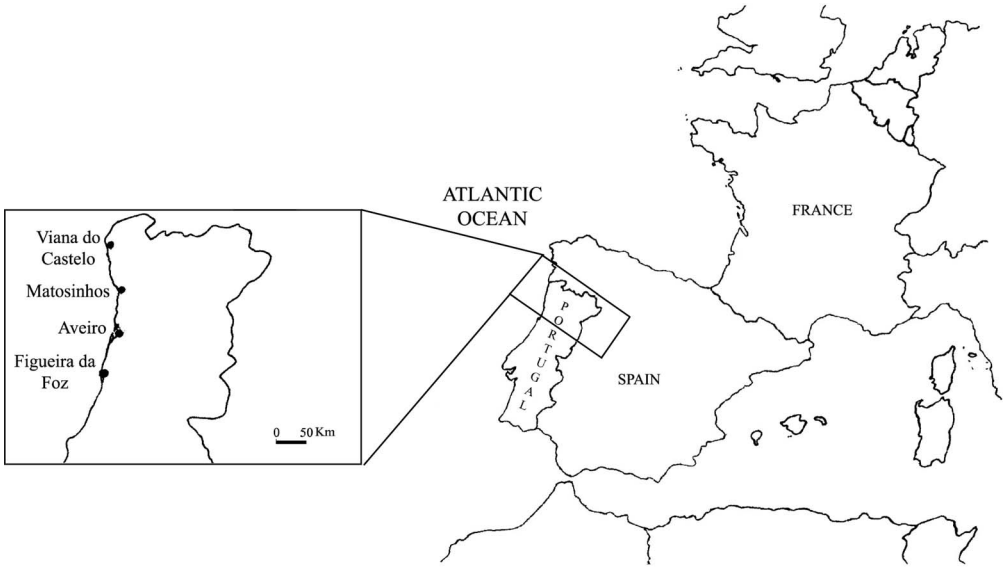

FIGURE 1. Geographic location of the 4 sampled areas (VC, Viana do Castelo; M, Matosinhos; A, Aveiro; and FF, Figueira da Foz) along the north-central Portuguese coast.

interval]) and mean intensity ± SD (range). Besides that, the first-order jackknife estimator of species richness (S_{JK}) rounded to the nearest integer and respective standard deviation values were evaluated using EstimateS software (Colwell, 2005).

Parasites of 7 different taxa were identified on the flounders examined: *Caligus diaphanus* von Nordmann, 1832, *Caligus* sp., and *Lepeophtheirus pectoralis* (Müller, 1777) (Copepoda: Caligidae); *Acanthochondria cornuta* (Müller, 1776) (Copepoda: Chondracanthidae); *Holobomolochus confusus* (Stock, 1959) (Copepoda: Bomolochidae); *Nerocila orbignyi* (Guérin-Méneville, 1832) (Isopoda: Cymothoidae); and praniza larvae (Isopoda: Gnathiidae) (Table II). Infected host specimens were quite common, varying from 21 fish (70 [51–85]%) off Viana do Castelo to 30 fish (100 [88–100]%) off Matosinhos, Aveiro, and Figueira da Foz. Multiple infections were more frequent off Matosinhos, with all the infected host specimens (30 fish/100 [88–100]%) harboring more than 1 parasite species, followed by Aveiro and Figueira da Foz (28 fish/93 [78–99]%), and Viana do Castelo (7 fish/23 [10–42]%). Copepod specimens were found on 7 (23 [10–42]%) fish off Viana do Castelo and all (30/100 [88–100]%) fish off Matosinhos, Aveiro, and Figueira da Foz. Isopods were found on 20 (67 [47–83]%), 3 (10 [2–27]%), and 1 (3 [0–17]%) fish off Viana do Castelo, Matosinhos, and Aveiro, respectively. In contrast to what was previously described for the northern Europe flounder populations, and similar to what was observed in the south-central Portuguese coast, neither *Lernaeocera branchialis* (L.) nor any monogenean species was found during our study.

The infection of the European flounder *P. flesus* off the north-central Portuguese coast by ectoparasitic metazoans seems to be quite common, judging by the total number of infected fish found in our study. Furthermore, copepods were the most frequent parasites, whereas the isopods occurred only on rare occasions. With the exception of *C. diaphanus*, *Caligus* sp., and *N. orbignyi*, which, as far as we know, are new host records, all the other species have already been recorded on flounders from the Atlantic Ocean, and from the North, Norwegian, and Baltic seas.

The number of parasitic species recorded varied across locations, ranging between 2 and 5. However, while in VC and FF the observed and estimated richness values coincided, in M and A they did not, thereby indicating that the true species richness for the latter locations is higher than the one observed in our survey. The minimum value documented for the observed species richness was recorded for *Lepeophtheirus pectoralis* and *A. cornuta*, 2 species common to all the sampled locations. In fact, prevalence and intensity values recorded for these 2 species suggest that they are probably common parasites of flounders throughout the north-central Portuguese coast. Both copepods were dominant off Matosinhos, Aveiro, and Figueira da Foz, whereas off Viana do Castelo the highest prevalence value was recorded for gnathiid pranizae. In the North Sea, *Lepeophtheirus pectoralis* and *A. cornuta* also appear to be common parasites of the European flounder (Boxshall, 1974; Schmidt, 2003). All other identified parasites, i.e., *C. diaphanus*, *Caligus* sp., *H. confusus*, and *N. orbignyi*, exhibited comparatively lower prevalence and total intensity values, indicating that they are probably not common in flounders from the studied area. For the latter 4 species, differences in host age may help to explain their diverse occurrence on the fish samples. Moreover, all were absent from FF, the sampling location where older fish, i.e., fish possessing higher mean total weight and length values, were collected. The absence of *Lernaeocera branchialis*, a parasite that can constitute a severe pest with significant economic impact (Kabata, 1979), is noteworthy, since this species is a common parasite on flounders from the North (Schmidt, 2003) and Baltic Seas (Køie, 1999). This result is probably related to the absence of the main definitive host species (gadoid fishes) from the area under study (Kabata, 1979; Svetovidov, 1986).

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Early Migration of *Sarcocystis neurona* in Ponies Fed Sporocysts

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ABSTRACT: *Sarcocystis neurona* is the most important cause of equine protozoal myeloencephalitis (EPM), a neurologic disease of the horse. In the present work, the kinetics of *S. neurona* invasion is determined in the equine model. Six ponies were orally inoculated with 250×10^6 *S. neurona* sporocysts via nasogastric intubation and killed on days 1, 2, 3, 5, 7, and 9 postinoculation (PI). At necropsy, tissue samples were examined for *S. neurona* infection. The parasite was isolated from the mesenteric lymph nodes at 1, 2, and 7 days PI; the liver at 2, 5, and 7 days PI; and the lungs at 5, 7, and 9 days PI by bioassays in interferon gamma gene knock out mice (KO) and from cell culture. Microscopic lesions consistent with an EPM infection were observed in brain and spinal cord of ponies killed 7 and 9 days PI. Results suggest that *S. neurona* disseminates quickly in tissue of naive ponies.

Equine protozoal myeloencephalitis (EPM) is a serious neurologic disease and *Sarcocystis neurona* is the most important cause (Dubey et al., 1991). *Sarcocystis neurona* has a 2-host life cycle, including a meat-eating definitive host, the opossums *Didelphis virginiana* and *Didelphis albiventris* (Dubey, Lindsay, Kerber et al., 2001; Dubey, Lindsay, Saville et al., 2001). There is a wide range of intermediate hosts, including the raccoon (Dubey, Saville et al., 2001), armadillo (Cheadle, Tanhauser et al., 2001), skunk (Cheadle, Yowell et al., 2001), sea otter (Dubey et al., 2002), and the domestic cat (Dubey and Hamir, 2000; Dubey et al., 2000; Turay et al., 2002). The horse is considered an aberrant intermediate host (Dubey, Lindsay, Saville et al., 2001). Schizonts and merozoites are the only stages known in the horse, and they are found only in the central nervous system (CNS) following an uncharacterized migratory route. Attempts to demonstrate *S. neurona* in tissues of horses fed sporocysts have been unsuccessful despite the fact that horses developed neurological signs (Fenger et al., 1997; Lindsay et al., 2000; Cutler et al., 2001; Saville et al., 2001; Sofaly et al., 2002). In the present article, we have attempted to follow the migration of *S. neurona* in tissues of ponies by orally inoculating them with large numbers of sporocysts and examining at shorter postchallenge intervals.

Eight seronegative ponies (Table I) were randomly assigned to treatment (n = 6) or control (n = 2) groups and housed in separate stalls. Neurologic examinations were conducted before the initiation of the project and daily there after, including the date of termination. The examinations were performed by a coauthor (S.M.R.). Physical examinations were also performed daily. On day 0, cerebral spinal fluid (CSF) and blood samples were collected from each horse, and treatment ponies were inoculated with sporocysts via nasogastric intubation with 250×10^6 sporocysts (25 ml) and 120 ml doses of phosphate buffered saline (PBS) to ensure complete dosing. The sporocysts were of the raccoon isolate SN 37-R and had been obtained from the intestines of the laboratory-raised opossums fed tissues of experimentally infected raccoons as described (Sofaly et al., 2002; Stanek et al., 2002).

Control ponies were given saline solution (25 ml) and 120 ml doses of PBS via the nasogastric tube. Disposable gloves and plastic boots were used upon entrance into the control ponies' stalls to avoid cross-contamination and were immediately discarded afterwards. An empty stall was maintained between the control ponies and treatment ponies as well. Blood for serology was collected daily (days 1–9) and for buffy coat culture on terminal dates. Treatment ponies were randomly assigned to serial killing on days 1, 2, 3, 5, 7, and 9 PI, and the control ponies were killed on days 3 and 9 PI. Ponies were humanely killed with an overdose of Euthasol euthanasia solution (Delmarva Laboratories, Midlothian, Virginia), and CSF was collected via the atlanto-occipital space at postmortem.

Necropsy was performed on all ponies. At necropsy, samples of lung, liver, mesenteric lymph nodes, and mesenteric artery were removed aseptically for *S. neurona* isolation. Additional tissue samples were fixed in 10% buffered formalin for routine microscopic examination, including the heart, lung, diaphragm, liver, spleen, adrenal gland, kidney, tongue, mesenteric lymph node, mesenteric artery, cecum, sciatic

TABLE I. *Sarcocystis neurona* in tissues of ponies detected by parasite isolation.

Pony	Day PI	Bioassay in KO mice				Cell culture
		MLN*	Liver	Lung	CNS†	
6459	1	3/5‡	0/5	ND§	0/5	Negative
6460	2	5/5	1/5	ND	0/5	Positive¶
6617‖	3	0/5	1/5#	ND	0/5	Negative
6453	3	0/5	0/5	ND	0/5	Negative
6570	5	0/5	4/5	2/2	0/5	Negative
6451	7	0/5	2/5	2/2	0/5	Positive¶
744	9	0/5	0/5	0/5	0/5	Positive**
742‖	9	ND	ND	ND	ND	Negative

* MLN, mesenteric lymph node.
† CNS, central nervous system.
‡ No. of mice with demonstrable *S. neurona* merozoites/no. of mice inoculated with pony tissues.
§ ND, not done.
‖ Negative control ponies.
Mouse died from bacterial meningitis; no parasites detected in its tissues.
¶ Positive in mesenteric lymph nodes.
** Positive in lung.

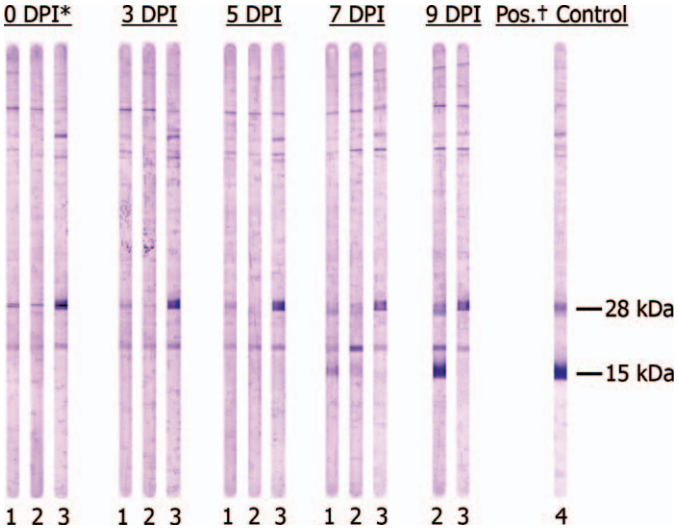

FIGURE 1. Reactivity of sera from ponies inoculated with *S. neurona* sporocysts. Immunoblots from SDS-PAGE separated non-reduced *S. neurona* proteins. The antigens were probed with sera serially collected beginning with preinoculation (day 0) and 3, 5, 7, and 9 DPI*. Negative control is pony no. 742 (lane 3), which did not receive sporocysts during the study. In all categories, lane 1 corresponds to pony no. 6451, lane 2 corresponds to pony no. 744, and lane 3 (control terminal) corresponds to pony no. 742. All samples were diluted at 1:10. Approximate molecular sizes are given at the margin in kDa. *DPI, days postinfection. †Positive control (lane 4).

nerve, and quadriceps. The entire small intestine was removed and cross-sectioned at the ileum, jejunum, and duodenum regions, and multiple sections from each region were placed in 10% buffered formalin. Finally, brain and spinal cord (with dura intact) were removed and sampled for protozoan isolation, followed by fixation in 10% buffered formalin for microscopic examination (Saville et al., 2004). All tissues were paraffin-embedded and sections were stained with hematoxylin and eosin (H & E) for microscopic evaluation. Histologic evaluation sampled the brain stem at 4 levels (thalamus, mesencephalon, metencephalon, and myelencephalon) and the cervical, thoracic, lumbar, and sacral spinal cord at 7, 7, 5, and 2 levels, respectively.

Sarcocystis neurona immunoblot analysis was used to detect IgM-specific antibodies in serum for ponies nos. 6451, 744, and 742 at preinoculation as well as at 3, 5, 7, and 9 days PI, and in CSF for all treatment ponies and 1 control pony (no. 742) preinoculation and at postmortem. The immunoblot was performed as previously described with slight modifications to incorporate the *S. neurona* antigen (isolate SN-UCD1) and facilitate IgM antibody detection (Granstrom et al., 1993; Marsh et al., 2001, 2004; Murphy et al., 2006). A positive control horse serum and CSF was used (Sofaly et al., 2002).

For isolation of *S. neurona* in cell culture, sections of brain, lung, liver, mesenteric lymph nodes, mesenteric artery, and spinal cord were immediately processed separately following necropsy as previously described (Dubey et al., 1991). The dura mater of spinal cord was opened, and 0.5 cm cross section segments of spinal cord representing spinal cord segments at C_{1-2}, C_{3-4}, C_{5-6}, C_7–T_1, T_{4-5}, T_{8-9}, T_{12-13}, L_{1-2}, L_{3-4}, and L_7–S_2 were removed. Tissue samples were washed with antibacterial/antifungal wash, placed in 4 C sterile phosphate solution, and stored at 4 C until ready for processing onto cell culture. The buffy coat from 10 ml whole blood was removed with a sterile pipette, mixed with 4 ml of sterile RPMI protozoal media 1640 (Gibco, Grand Island, New York), and then inoculated onto a monolayer of equine dermal cells for protozoal isolation (Dubey et al., 1991). The cells were allowed to culture for 88–97 days (Dubey et al., 1999). Positive cell cultures were determined by inverted microscope followed by confirmation of merozoite presence in media using cytocentrifugation, followed by Dif-Quick staining (IMEB, Inc., San Marcos, California) to observe merozoites.

Tissues (Table I) were shipped on ice to the USDA laboratory (Beltsville, Maryland) for bioassay in interferon gamma gene knock out (KO) mice (BALB/c-Ifng^{tm1Ts}, females, 6 to 12 wk old, Jackson Laboratories, Bar Harbor, Maine). Tissues were homogenized in 0.85% sodium chloride aqueous solution and inoculated s.c. into KO mice (Sofaly et al., 2002). Mice were observed for 60 days unless they exhibited neurologic symptoms, at which point they were killed, and their cerebellum processed for *S. neurona* immunohistochemistry (IHC). Selected pony tissue samples were also stained by IHC for *S. neurona* antigens as previously described by Dubey and Hamir (2000).

All ponies appeared to be clinically normal at all postinoculation intervals, aside from some nonspecific physical signs of illness, such as fever and nasal and ocular discharge. Neurologic deficits were not noted in any of the ponies throughout the study.

Sarcocystis neurona was isolated from tissue of the ponies by bioassay in KO mice and was isolated in cell culture from 2 ponies (Table I). Differences in results most likely are due to differences in the sensitivities of each test and parasite distribution within each tissue. *Sarcocystis neurona* was isolated from mesenteric lymph node at 1, 2, and 7 days PI; liver at 2, 3, 5, and 7 days PI; and lung at 5, 7, and 9 days PI. *Sarcocystis neurona* was not found in any pony tissues sections stained with H & E or with *S. neurona* antibody. Additionally, only serum from pony no. 744 (killed 9 days PI) had IgM antibodies specific to *S. neurona* at 2 markers, 28 kDa and 15 kDa (Fig. 1).

Histologic tissue sections were examined by 2 coauthors (J.P.D. and M.J.O.). Lesions associated with protozoan infections were not found in ponies killed 1–5 day PI. Ponies killed at 7 and 9 days PI had foci of inflammation that are consistent with EPM infection. Pony no. 6451 had a single focus of mild severity in the C_{3-4} region of the spinal cord. The focus had moderate gliosis, but perivascular leukocytes were not observed. Pony no. 744 had multiple lesions of mild to moderate severity observed in the cervical intumescence (C_7–T_1) and T_{12-13} regions of the spinal cord, with foci of lesser severity in the thalamus, mesencephalon, and metencephalon (pons region). The lesions within the spinal cord consisted of moderate focal perivascular astro- and microgliosis with mononuclear cell infiltrate in the grey matter. The lesions within the brain were restricted to the grey matter and included focal glial nodule formation and perivascular increases in glial density. However, no parasites were detected by *S. neurona*–specific IHC staining on tissues with lesions compatible with protozoal infection.

Once it is ingested, the 2 possible routes that a parasite can take to reach distant organs are via the lymph, the portal blood, or both (Dubey et al., 1989). In KO mice, the first generation of *S. neurona* schizogony occurs intravascularly in visceral tissues before invasion of the CNS. Mesenteric lymph node invasion is evidence of lymphatic dissemination, and liver and lung infections suggests hematogenous spread after oral inoculation. Although parasitemia was not directly evident within

this study, parasite isolation from the liver and lung suggest a hematogenous dissemination of the parasite. In the present study, seroconversion for *S. neurona–specific* IgM was detected at 9 days PI, and this finding is confirmed in previous equine studies that have reported IgG immunoconversion at 8 days PI (Lindsay et al., 2000; Sofaly et al., 2002). Finally, although none of the ponies in this study had detectable anti-*S. neurona* IgM antibodies in the CSF by 9 days PI, that finding is supported by similar findings in previous equine models, where the earliest reported CSF conversion occurred at 28 days PI (Fenger et al., 1997).

The results of the present study, and those in KO mice and in raccoons (Dubey, 2001a, 2001b; Stanek et al., 2002) fed sporocysts, indicate that *S. neurona* quickly travels from the gastrointestinal tract to the lymph nodes, within 24 to 48 hr. Mesenteric lymph node invasion is evident as early as 24 hr PI, and liver infection occurs as early as 2 days PI. It is interesting to note that the only other time that *S. neurona* was isolated from the blood after oral inoculation of sporocysts at 21 days PI from a severe immunocompromised disease (SCID) horse (Long et al., 2002). Our results suggest that parasitemia occurs at or before 2 days PI (as is evident in liver bioassays). Although *S. neurona* was not isolated from brain and spinal cord of the ponies, histologically, lesions in the CNS of ponies at day 7 and 9 PI were suggestive of an *S. neurona* invasion based upon characteristic tissue changes, correlation to isolation from other tissues in KO mice or tissue culture, and comparable kinetics in previously reported KO mice and raccoon studies. This is the first time that *S. neurona* has been isolated from the mesenteric lymph nodes, liver, and lungs from either experimentally or naturally infected immunocompetent equids.

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A Human Case of *Plagiorchis vespertilionis* (Digenea: Plagiorchiidae) Infection in the Republic of Korea

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ABSTRACT: *Plagiorchis vespertilionis* (Digenea: Plagiorchiidae) is generally considered a bat parasite, but here it is reported for the first time in a human. The patient was a 34-yr-old male who lived in a coastal village of Haenam-gun (county), Jeollanam-do (province), Republic of Korea. Only 1 worm, 2.6 mm long and 0.7 mm wide, was recovered after praziquantel treatment and purging with magnesium salts. The fluke was characterized by a large body size, a sucker ratio of 1:1, a straight cirrus organ, a short distance between the ventral sucker and ovary, well-developed vitellaria, a uterus with descending and ascending loops, and fully developed eggs with an average size of 32.5 × 17.5 μm. The patient had habitually eaten the raw flesh of snakehead mullet and gobies that had been caught near his village. The present case represents the first record of a human *P. vespertilionis* infection.

Species of *Plagiorchis* Lühe, 1899 (Digenea: Plagiorchiidae) are intestinal parasites of reptiles, birds, and mammals (Yamaguti, 1958), and their metacercariae are found in mosquito larvae, insect naiads, freshwater snails, and freshwater fish (Tanabe, 1922; Asada et al., 1962; Komiya, 1965; Hong et al., 1996; Chai and Lee, 2002). Human infections of *Plagiorchis* spp. are rare. Worldwide, only 11 natural human cases have been reported in the literature (Radomyos et al., 1989; Hong et al., 1996). The species responsible for human infections include *Plagiorchis philippinensis* in the Philippines (Sandground, 1940), *Plagiorchis muris* Tanabe, 1922 in Japan and the Republic of Korea (Asada et al., 1962; Hong et al., 1996), *Plagiorchis javensis* in Indonesia (Sandground, 1940), and *Plagiorchis harinasutai* in Thailand (Radomyos et al., 1989). In addition to *P. muris* in Korean house rats (Seo et al., 1964, 1981), 7 other species have been reported in Korean bats, i.e., *Plagiorchis vespertilionis* (Müller, 1780) Braun, 1900, *Plagiorchis magnacotylus* Park, 1939, *Plagiorchis orientalis* Park, 1939, *Plagiorchis rhinolophi* (Park, 1939), *Plagiorchis koreanus* Ogata, 1938, *Plagiorchis kyushuensis* Kifune and Sawada, 1979, and *Plagiorchis corpulentus* Kifune and Sawada, 1979 (Park, 1939a, 1939b; Sogandares-Bernal, 1956; Kifune et al., 1983).

Plagiorchis vespertilionis was first described in the brown long-eared bat *Plecotus auritus* in Europe, and then in many countries, including the Republic of Korea (Sogandares-Bernal, 1956). A subspecies, named *Plagiorchis vespertilionis parorchis*, was described in the United States (Macy, 1960). The xiphidiocercariae of *P. vespertilionis parorchis* develop in the snail intermediate host, *Lymnaea stagnalis* and encyst in mosquito larvae, caddis-fly larvae, mayfly larvae, and dragonfly nymphs (Macy, 1960). In addition, an attempted experimental infection of mice and hamsters with this species was successful, and adult flukes were harvested from the small intestine (Macy, 1960). During studies

TABLE I. Morphological characteristics of *Plagiorchis vespertilionis* in the present specimen and those reported by previous workers.

Organs	The present specimen	Sogandares-Bernal (1956)	Tkach et al. (2000)
Body length × width	2.6 × 0.7 mm	2.7–4.8 × 0.8–1.6 mm	2.3–4.1 × 0.2–0.5 mm
Sucker ratio	1.0:1.0	1:0.67–1:1.39	1.0:1.2
Pharynx	0.14 × 0.15 mm	0.11–0.19 × 0.11–0.15 mm	Diameter: 0.1 mm
Esophagus	Nearly absent	Variable in length or absent	Very short or none
Ceca	Extend to near posterior extremity	Extend into posterior end of body	End 0.18 mm from body extremity
Cirrus sac	Extend from VS* to anterior part of ovary	Extend from VS to anterior part of ovary	Elongated and situated along the median body axis
Cirrus	0.195 × 0.057 mm straight form	Not mentioned	0.38–0.55 × 0.013–0.015 mm, coiled or straight form
Testis	Postovarian, oval Anterior testis: 0.184 × 0.2 mm Posterior testis: 0.23 × 0.23 mm	Rounded, postequatorial, oblique Anterior testis: 0.21–0.53 mm Posterior testis: 0.23–0.50 mm	Postovarian, oval, and oblique Anterior testis: 0.26–0.33 × 0.15–0.23 mm Posterior testis: 0.25–0.35 × 0.16–0.24 mm
Ovary	Postacetabulum 0.163 × 0.213 mm	Rounded, postacetabulum 0.17–0.27 mm	Oval or rounded, median of cirrus sac 0.14–0.26 × 0.10–0.21 mm
Vitellaria	Extend to posterior end of worm	Distributed lateral to ceca and extend to posterior end of worm	Well developed and distributed on either side of body
Uterus	With descending and ascending loops	With descending and ascending loops, intertesticular	With descending and ascending loops
Eggs	32.5 ± 1.3 × 17.5 ± 0.5 μm	37 × 18 μm	29–34 × 16–21 μm

* Ventral sucker.

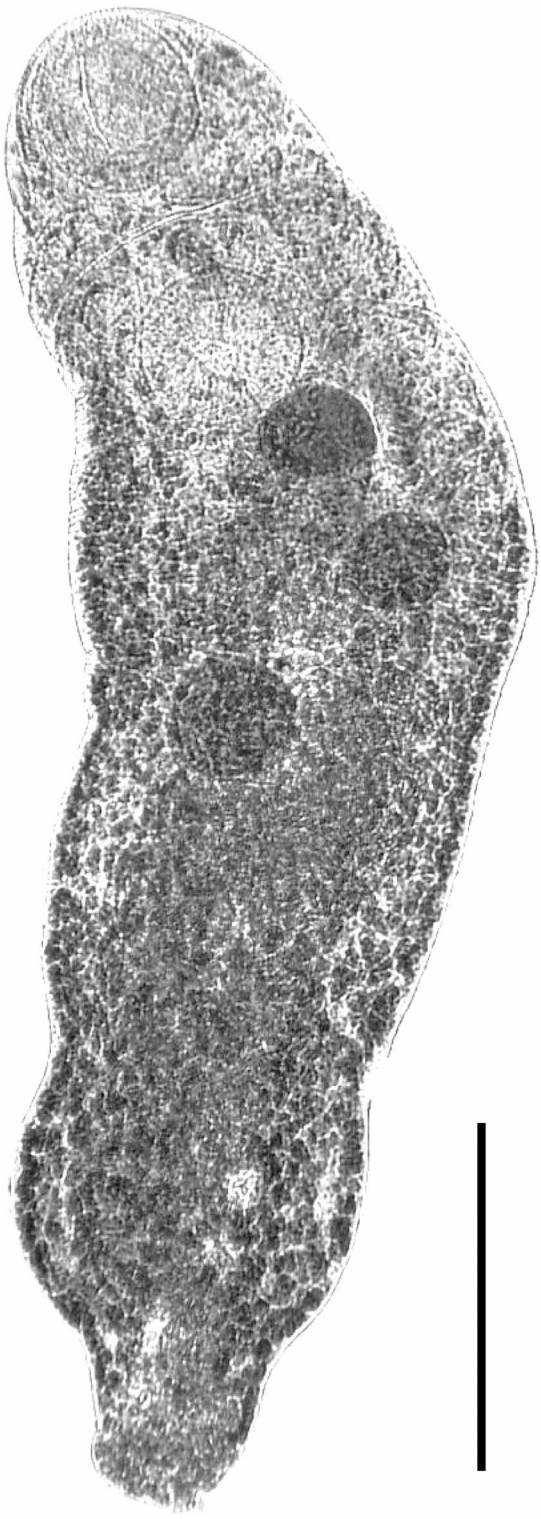

FIGURE 1. *Plagiorchis vespertilionis* adult specimen collected from the described case. Acetocarmine stain. Scale bar = 0.6 mm.

on intestinal fluke infections among residents of southern coastal areas of the Republic of Korea, we discovered a human case naturally infected with *P. vespertilionis*, which is the first confirmed case of natural human infection by the parasite.

The southern coastal area (Haenam-gun, Jeollanam-do) of the Republic of Korea was surveyed for human intestinal parasites between August and September 2003. Fecal samples were collected from residents and examined for helminth eggs and protozoan cysts using the Kato thick smear and formalin-ether sedimentation techniques (Beaver et al., 1984). Results revealed a helminth egg or protozoan cyst prevalence of 37.9%; a considerable number of people were infected with *Gymnophalloides seoi* Lee, Chai and Hong, 1993, heterophyid flukes, and other parasites (data not shown).

To identify the adult flukes, people shedding presumed gymnophallid and heterophyid eggs (n = 11) were treated orally with 10 mg/kg of praziquantel in a single dose and purged with 30 g of magnesium sulfate. Subsequently, diarrheic stools were collected and examined under a stereomicroscope. Numerous adult specimens of gymnophallid and heterophyid flukes were recovered. These were fixed in 10% neutral buffered formalin under coverslip pressure, stained with Semichon's acetocarmine, and mounted in balsam. Flukes were then individually identified under an optical microscope. One specimen of *Plagiorchis* sp. was recovered from a 34-yr-old man. The worm was an adult fluke with numerous uterine eggs. Based on morphological characteristics in Table 1, this specimen was identified as *P. vespertilionis* (Sogandares-Bernal, 1956; Tkach et al., 2000). The specimen has been deposited in U.S. National Parasite Collection (Beltsville, Maryland) under the accession number USNPC 99592.

In previously reported human cases of *Plagiorchis* sp. infection, a small number of flukes have usually been found among a large number of other intestinal or liver flukes (Sandground, 1940; Asada et al., 1962; Radomyos et al., 1989; Hong et al., 1996). Interestingly, no specific gastrointestinal symptoms were recorded in these cases. Similarly, the present case, involving infection with only 1 *P. vespertilionis* and 9 *Heterophyes nocens* specimens, had no intestinal symptoms of note. Thus, it appears that a small number of *Plagiorchis* sp. is incapable of causing a significant pathological condition (McMullen, 1937).

In terms of the identification of *Plagiorchis* species, the location and morphologies of the cirrus sac, cirrus, testes, ovary, and Mehlis gland, and the positions and extents of vitellaria and uterus, and the sucker ratio are important (Sandground, 1940; Sogandares-Bernal, 1956; Kifune et al., 1983, 1997; Radomyos et al., 1989; Tkach et al., 2000). However, the taxonomic status of *Plagiorchis* spp. occurring in European bats was confusing because of the high degree of morphological similarity between different forms and species and an inadequate initial description of *P. vespertilionis* by Müller in 1780 (Tkach et al., 2000). In particular, the morphological data on the *P. vespertilionis* group, i.e., *P. vespertilionis*, *Plagiorchis muelleri* Tkach and Sharpilo, 1990, and *P. koreanus* Ogata, 1938, were inadequate. To resolve this problem, Tkach et al. (2000) used molecular (nuclear rDNA ITS region) techniques, and confirmed the distinct characters of these 3 species.

Our specimen differs from similar species in the following ways. It differs from *P. muris* reported in a human (Hong et al., 1996) and in house rats in the Republic of Korea (Seo et al., 1964), in the less extensive distribution of vitellaria, from the posterior body extremity only to the ventral sucker median; of the locations of ovary and testes; and of smaller egg size. Our fluke has a sucker ratio of 1:1, which differs from *P. koreanus*, which has a smaller ventral sucker (Ogata, 1938; Kifune et al., 1997). *Plagiorchis muelleri* differs from our specimen in having a substantially greater distance between the ovary and ventral sucker (Tkach et al., 2000).

The known second intermediate hosts of *P. vespertilionis*, i.e., caddisfly larvae, mayfly larvae, and dragonfly nymphs, have been suggested to be possible sources of natural human infection (Macy, 1960). However, in the present study, the patient recalled he had eaten raw freshwater fish, including snakehead mullet and goby. Thus, the source of infection in this patient remains uncertain. In a recent study of *P. muris* in the Republic of Korea, freshwater fish were considered the source of human infection (Hong et al., 1996). In addition, it was shown that dragonflies play a significant role as a second intermediate host for *P. muris* (Hong et al., 1998, 1999). In view of the environment in which the present patient lived, there is a possibility that freshwater fish and freshwater snails may be second intermediate hosts for *P. vespertilionis*. Studies are required to elucidate the whole life cycle of *P. vespertilionis* in the Republic of Korea.

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Influence of a Thermal Discharge on Parasites of a Cold-Water Flatfish, *Pleuronectes americanus*, as a Bioindicator of Subtle Environmental Change

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ABSTRACT: A study was conducted to ascertain the influence of a thermal discharge on the health and parasites of a coastal cold-water flatfish, the winter flounder (*Pleuronectes americanus*), a species sensitive to environmental change. Flounder were sampled in spring 1998 and 1999 beneath the plume and at reference sites north and south up to 1 km from the discharge. Species diversity and estimates of abundance of macroscopic algae, invertebrates, and fish were also recorded. After capture by scuba divers, a comparison of condition factor, organ indices, blood values, histology, and parasites was made between groups of fish from the discharge and reference sites. Diversity and abundance of algae, invertebrates, and fish were considerably greater beneath the plume than at the reference sites. The thermal water had no apparent effect on flounder taken beneath the plume, but it affected both its ecto- and endoparasites. Prevalence and mean abundance of *Cryptocotyle lingua* metacercariae were significantly greater, whereas *Trichodina jadranica* and *Gyrodactylus pleuronecti* were less on the gills of fish sampled beneath the plume than at the reference sites. Four endoparasites, i.e., *Ceratomyxa drepanopsettae*, *Steringophorus furciger*, *Macvicarius soleae*, and *Lecithaster gibbosus* were significantly more abundant in the reference samples. These results suggest that environmental change affected transmission of the parasites of winter flounder exposed to the thermal effluent.

Coastal fossil fuel-generating plants discharging heated water into the sea tend to have some influence on the environment and also on benthic communities, which invariably are sessile or possess limited scope for movement. Some studies have reported changes in abundance and spatial variability of benthic assemblages by thermal effluent affecting the most abundant species, whereas others have observed minimal changes in the vicinity of the discharge (Landicci et al., 1999). It seems that thermal tolerance of some species is related to the temperature; from 7 to 10 C above the ambient impacted on benthic organisms more than 1–2 C (Suresh et al., 1993; Landicci et al., 1999). Additionally, the volume of water, season, temperature, and rate of discharge could also have a profound influence on benthic organisms (Landicci et al., 1999).

Several methods have been proposed to assess the impact of environmental change in fish (Adams, 1990). These include external abnormalities, condition factor, organ indices, and histology in species that are sessile and sensitive to ecosystem alterations (Adams, 1990). More recently, there is evidence that some fish parasites can be useful as bioindicators of stress (Khan and Thulin, 1991). A thermal power plant in Holyrood, Newfoundland, Canada, using Bunker oil as fuel has been discharging heated seawater into the ocean for a number of years where a population of winter flounder (*Pleuronectes americanus*), a species sensitive to environmental change, occurs. The influence of the discharge was reported to affect the horse mussel (*Modiolus* sp.), but noth-

ing was known of its influence on fish health (LGL Ltd., 1999). Consequently, a study was designed to investigate the effect of the thermal discharge at Holyrood on winter flounder living beneath the plume and to compare the results with samples taken from fish at varying distances north and south from the discharge. This included some of the biological indicators mentioned above, including blood values of the flounders and also its parasites.

The thermal generating plant, using Bunker AC as fuel oil, is located at Holyrood (47°27′N, 53°08′W). Approximately 6×10^5 L/min of thermal water, 5–13 C, was discharged into Conception Bay, but temperature change extended up to only 1 m below the surface (LGL Ltd., 1999). The benthic water temperature during both years was 0 C, whereas the surface temperatures were 3–4 C in May and 7–8 C in June. Benthic sampling was conducted at all sites for macroscopic algae, invertebrates, and fish, and estimates of their abundance were recorded. Winter flounder were captured beneath the plume by scuba divers using a fish-landing net at about 5 m in late May and June 1998 and in May 1999. In addition, flounder were taken at 0.5 m north (upcurrent) and downflow at 0.4–0.5 and 1.0 km southwest from the discharge in 1998 and downflow at 100–200 and 400 m in 1999. The fish were examined after capture for external lesions and macroscopic parasites, which were enumerated. Two skin smears were prepared for examination subsequently for microscopic parasites. Blood was taken from the caudal vein by a needle and heparinized syringe to determine hermatocrit, hemoglobin, and total plasma protein in the serum as reported in another study (George-Nascimento et al., 2000). A blood smear was prepared for ascertaining lymphocytic levels after staining with Giemsa. At necropsy, the total body length, eviscerated body, liver, and gonad masses were examined. Samples of tissues, including gill, liver, spleen, and gonad were fixed in 10% buffered formalin and processed by conventional histological methods. Cross sections, 10 μm, were stained with hematoxylin and eosin. Sections of spleen were also stained with Perl's Prussian blue method for hemosiderin that was estimated by digital image analysis (Khan and Nag, 1993). Bile was taken from the gallbladder by a needle and syringe, preserved with an equal volume of 70% ethanol, and examined subsequently for myxozoans microscopically (×400). The digestive tract was examined for metazoan parasites that were removed and fixed in 70% ethanol. Digenes were stained with borax carmine. Metacercariae infecting the gills were estimated in cross sections after microscopic examination of 10 secondary lamellae.

Samples of flounder were compared statistically according to sex, site, and year of capture. Variables compared included condition factor (eviscerated body mass/length^3), hepatosomatic index (liver mass/eviscerated body mass), gonadosomatic index (gonadal mass/eviscerated body mass), hemoglobin (g%), hematocrit (%), total plasma protein (g%), and lymphocyte levels (no. of lymphocytes/1,000 erythrocytes in Giemsa-stained blood smears; see George-Nascimento et al., 2000). However, the samples examined in 1999 at 100 and 200 m from the discharge were pooled, with the exception of the parasites in the digestive tract, because of the paucity in numbers of fish that were captured. The data were analyzed using an analysis of covariance for site significance. These included condition (k) factor (eviscerated body mass/length^3), organ indices (organ mass/eviscerated body mass), and blood values. Prevalence (%) and mean abundance were calculated for each fish group. Chi-square and Fisher's exact probability tests compared prevalence, whereas the nonparametric test of Kruskal–Wallis, after log transformation of parasitic numbers, was used for comparing fish groups according to site.

Profound differences in the diversity and abundance of the species and genera of algae, invertebrates, and fish occurred between the site at the thermal outfall and locations north and south of it. Three species of seaweed (*Callithamnion*, *Bryopsis*, and *Gloiosiphonia*) were confined to the plume, 7 species within 5 genera (*Chondrus*, *Fucus*, *Saccharina*, *Cladophora*, and *Scytsiphon*) were observed near the plume, whereas only 5 species (*Alaria*, *Laminaria*, *Spongomorpha*, *Urospora*, and *Monostroma*) occurred north and south of it. Thirteen species of invertebrates, including 7 species of grazing crustaceans (*Semibalanus*, *Gammarus*, *Calliopius*, *Ischyroceros Jassa*, *Jaera*, and *Idothea*), a crab (*Cancer* sp.), 4 species of mollusks (*Littorina*, *Nucella*, *Lacuna*, and *Mytilus*), and an echinoderm (*Asterias* sp.) were present near the plume, whereas only a crab (*Hyas* sp.), 2 mollusc species (*Modiolus* and *Buccinum*), and an echinoderm (*Strongylocentrotus* sp.) occurred at the other sites. Large numbers of *Littorina littorea*, a shallow-water mussel, covered the bottom of the effluent channel. Additionally, 9 species of fish (*Salmo trutta*, *Tautogolabrus adspersus*, *Myoxocephalus octodecemspinosus*, *M. scorpius*, *Macrozoarces americanus*, *Pholis gunnellus*, *Gasterosteus aculeatus*, *Microgadus tomcod*, and *Pleuronectes americanus*) were observed near the plume, in contrast to 4 species (*Stichaeus punctatus*, *Lycodes lavalei*, *Hippoglossoides platessoides*, and *P. americanus*) at the other locations. Rating the abundance of seaweeds, invertebrates, and fish species between the reference sites and the plume, the latter exceeded the others by a magnitude of 15 to 20. The prolific growth of the seaweeds and abundance of animals declined beneath the plume after the plant ceased operations during summer and as the area came under the influence of colder, ambient seawater. However, growth and abundance returned to normal once the plant resumed operations in autumn. The flora and fauna near the plume have remained unchanged since an initial survey was conducted in 1976 (R. G. Hooper, unpubl. obs.). In summary, a greater diversity and abundance of these organisms were evident near the plume than at locations north or south of it.

The stomachs of foraging winter flounder sampled during 1998 and 1999 below the plume contained crustacean remnants, especially gammarid amphipods, and, less often, small molluscs, mainly gastropods. Flounder sampled during May in both 1998 and 1999 at the reference sites exhibited no evidence of feeding, and, at the time of capture, they lay covered by sediment at the bottom. Samples of flounder taken in June 1998 fed on polychaetes, crustaceans, and molluscs at the downstream sites, and the fish were located lying above the sediment.

Comparison of biological variables, i.e., condition factor and organ indices of male and female winter flounder sampled below the plume and reference sites, 0.4–0.5 north and south to 1.0 km away, revealed no significant differences. However, there was an exception: the gonadal somatic index of males sampled <0.5 m from the discharge point was significantly greater than that of the other 2 groups. Gonad development was delayed because males of the other groups had produced sperm, whereas examination of the testes of the aforementioned group revealed spermatids in the seminiferous tubules. Blood values, including hematocrit, hemoglobin, total plasma protein, and lymphocytic levels, were similar in all groups of fish. Additionally, comparison of the variables of flounder sampled in 1999 near the plume and at 100–200 and 400 m exhibited no differences. Examination of histological sections of the gills, liver, spleen, and gonads revealed an absence of lesions in all groups of fish in 1998 and 1999, excepting for an infection with metacercariae of the digenean *Cryptocotyle lingua*, which caused distortion of the secondary gill lamellae.

There were significant differences in the prevalence of some parasites infecting winter flounder from the 3 sites in 1998 and 1999. *Trichodina jadranica* (=*murmanica*) (Ciliophora; see Arthur et al., 2004), *Ceratomyxa drepanopsettae* (Myxozoa), *Gyrodactyus pleuronecti* (Monogenea), *Steringophorus furciger*, *Macvicarius soleae*, and *Lecithaster gibbosus* (Digenea) were significantly greater in flounder sampled at the reference sites than beneath the plume (Table I). Although the prevalence of *C. lingua* was similar at the 3 sites, the mean abundance on the skin and in the gill lamellae of fish sampled at the plume was significantly greater than at various distances away from it (Table II). This is not unusual, because Sekar and Threlfall (1970) observed that an infection with *C. lingua* was associated with the abundance of its molluscan intermediate host, the periwinkle (*Littorina littorea*), which occurred in greater numbers in the intertidal warm water than in other areas. Additionally, the mean abundance of *S. furciger* was significantly less in samples taken below the plume in both years of sampling than further away (Fig. 1). The abundance of *M. soleae* and *L. gibbosus* was consistently low (<1.0), except in 1998 at 400 m, when that of *M. soleae* was 1.2 ± 0.2. No nematodes or acanthocephalans were observed in the digestive tract.

The present study focused on winter flounder, a sedentary, cold-water species that has been shown to be sensitive to environmental change (Khan, 2003, 2004). It was anticipated that this fish would respond to an environmental disturbance associated with the discharge of thermal effluent. The results from the present study, based on several conventional bioindicators, indicate that the heated effluent caused no effects on fish health compared with samples from 2 reference sites in 1998 and 1999. However, differences in the prevalence and abundance of some parasites suggest that subtle changes in environmental conditions probably occurred that affected its parasites. Two ectoparasites attached

TABLE I. Prevalence (%) of parasites* infecting winter flounder (pooled sexes) north (upcurrent, uc), and south (downcurrent, dc) (m) from a site discharging thermal effluent at Holyrood, Newfoundland, Canada, in 1998 and 1999.

		Parasite/prevalence (%)							
Yr	Site/m	n	Tr	C	G	Cl	St	McV	L
1998	500 (uc)	27	100	100	100	52	100	37	41
	Plume	35	35†	14†	9†	100†	31†	36	3
	400–500 (dc)	51	96	100	100	82	100	80†	26
	1,000 (dc)	29	100	100	97	72	100	55	62†
1999	500 (uc)	35	100	100	100	28	100	46	54
	Plume	15	20†	13†	13†	100†	13†	8†	0
	100–200 (dc)	26	100	100	93	86	100	35	20
	400 (dc)	14	100	100	100	79	100	65	71†

* Tr = *Trichodina*, C = *Ceratomyxa*, G = *Gyrodactylus*, Cl = *Cryptocotyle lingua*, St = *Steringophorus*, McV = *McVicarius*, L = *Lecithaster.*
† Significantly different ($P \leq 0.05$) from comparison group(s).

to the gills, *T. jadranica* and *G. pleuronecti*, are known to be sensitive to high temperature and to decline under these conditions (Barker et al., 2002; Khan, 2004). Another ectoparasite in the skin, *C. lingua*, favors warmer water as its abundance increases during summer (Sekar and Threlfall, 1970). Differences in the prevalence of 4 endoparasites, i.e., *C. drepanopsettae* and 3 digeneans, i.e., *S. furciger*, *M. soleae*, and *L. gibbosus*, might also have been influenced by subtle environmental changes. This is supported by the observation that the growth of some species of algae, especially *Fucus* spp., was prolific and associated with an abundance of gammarid amphipids, other invertebrates, and fish beneath the plume compared with minimal algal growth and a paucity of other organisms at the reference sites.

Thermal effluent has been reported to influence both the prevalence and mean abundance of parasites of fish, more often positively than negatively. Lafferty (1997) listed 9 positive, 1 neutral, and 5 negative cases. In most discharges of heated water into freshwater systems, water temperature increments varied from 2 to 10 C. Esch et al. (1976) observed direct correlations among thermal loading, decreased body condition, and elevated prevalence of a ciliate, *Epistylis* sp., and a bacterium, *Aeromonas hydrophila*, in centrarchid fish. Parasitic abundance of a larval digenean was reported by Eure and Esch (1974) to be greater in large mouth bass, *Micropterus salmoides*, living in heated effluent, whereas Hirshfield (1983) observed that larval nematodes of *Eustrongylides* sp. in the mummichog, *Fundulus heteroclitus*, increased 4-fold in contrast to reference fish. Aho et al. (1982) noted also changes in distribution and abundance of 2 larval trematodes in mosquito fish, *Gambusia affinis*, living in a reservoir receiving thermal effluent from a nuclear reactor. Pojmanska and Dzika (1987) reported the most dramatic changes in a Polish lake receiving thermal effluent where 5 fish parasitic species disappeared, 3 new species became established, 4 species decreased in frequency, and 6 species increased in abundance. These changes resulted in a modification of dominance in the community structure. They attributed these changes to the habitat that affected both the invertebrate intermediate and avian definitive hosts. An unusual observation was noted by Sankurathri and Holmes (1976) in a lake where thermal water kept it ice-free in winter, and, as a result of waterfowl concentration, the prevalence of metacercariae infection in snails was approximately 75.8% compared with 6.5% at the reference site, but this was reversed during summer. Boxrucker (1979) reported that thermal effluent had little effect on parasites of a bullhead (*Ictalurus melas*), excepting an acanthocephalan, *Pomphorhynchus bulbocolli*, which showed a seasonal cycle at the reference site, but not at the outfall area. Camp et al. (1982) observed a decline in prevalence and abundance of metacercariae of *Ornithodiplostomum ptchocheilus* in the mosquito fish (*Gambusia affinis*) inhabiting a thermal reservoir in South Carolina when the summer temperature was high and also in winter. It was observed that the thermal effluent affected cercariae shedding and recruitment, eventually causing a decline. However, higher levels of parasitism occurred in spring in the reservoir than at the reference site. Camp et al. (1982) also noted that cercariae shedding from the infected

TABLE II. Mean abundance ($\bar{x} \pm$ SE) of metacercariae of *Cryptocotyle lingua* on the skin and in the secondary lamellae of the gills of winter flounder (pooled sexes) sampled north (upcurrent, uc) under a thermal plume at Holyrood, Newfoundland, Canada, and at 2 southerly (downcurrent, dc) sites in 1998 and 1999.

Yr	Location from discharge (m)	n	Skin	Gills
1998	500 (uc)	45	3.6 ± 0.4	1.3 ± 0.2
	0	35	79.0 ± 8.8*	2.8 ± 0.3*
	400–500 (dc)	51	6.4 ± 0.7	1.1 ± 0.2
	1,000 (dc)	29	4.0 ± 0.4	1.2 ± 0.2
1999	500 (uc)	63	2.8 ± 0.3	0.9 ± 0.1
	0	15	86.0 ± 14.0*	3.8 ± 0.5*
	100–200 (dc)	26	9.4 ± 1.1	0.8 ± 0.1
	400 (dc)	14	7.3 ± 1.0	0.6 ± 0.1

* Significantly different ($P \leq 0.05$) than other groups.

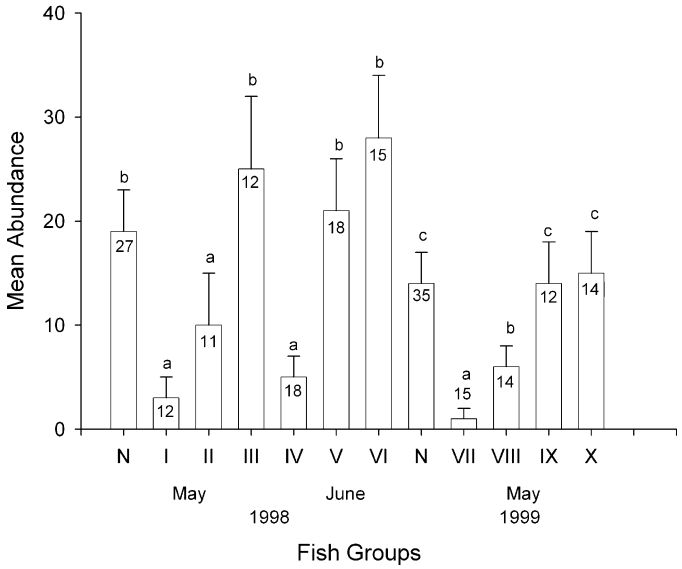

FIGURE 1. Mean abundance (0 ± SE) of *Steringophorus furciger* (Digenea) in the digestive tract of winter flounder (sexes pooled) sampled north (N), below the thermal plume (I, IV, VII) in 1998 and 1999 and at 100 m (VIII), 200 m (IX), 400 m (X), 500 m (II, V) and 1,000 m (III, VI) south of the discharge. Comparison groups with dissimilar letters are significantly different ($P \leq 0.05$).

snails (*Physa* sp.) continued throughout the winter. MacKenzie et al. (1995) cited an abstract of Hoglund and Thulin who reported no change in parasitism.

In summary, exposure of a cold-water, benthic, sedentary flatfish to thermal effluent discharged 5 m above the plume apparently caused no effect on body condition, organ indices, or hematological or pathological alterations. However, prevalence, or mean abundance, or both of some parasites of the fish were affected below the plume, 1 species increasing and 6 species declining. This observation suggests either sensitivity to the effluent or interruption of transmission via their intermediate hosts near the thermal plume. These results provide additional evidence to support the view that some parasites of fish can be useful as bioindicators of subtle environmental changes.

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Species of Coccidia (Apicomplexa: Eimeriidae) Infecting Pikas From Alaska, U.S.A. and Northeastern Siberia, Russia

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ABSTRACT: Eighty-eight fecal samples from 2 species of pika, *Ochotona collaris* and *Ochotona hyperborea,* collected in Alaska (N = 53) and Russia (N = 35), respectively, were examined for the presence of coccidia (Apicomplexa: Eimeriidae). Five oocyst morphotypes were observed. In *O. collaris,* we found *Eimeria calentinei, Eimeria cryptobarretti,* and *Eimeria klondikensis,* whereas in *O. hyperborea,* we found *Eimeria banffensis, E. calentinei, E. cryptobarretti, E. klondikensis,* and *Isospora marquardti.* This study represents new geographic records for all 5 coccidia and new host records for *E. cryptobarretti* and *I. marquardti.* Only minor quantitative differences were seen between the

sporulated oocysts we studied and those reported in their original descriptions.

Pikas are holarctic lagomorphs composed of the single genus, *Ochotona,* with 30 species (Wilson and Reeder, 2005). The majority of species are found in Asia, mainly in the Tibet (Qinghai-Xizang) Plateau region, but also in Afghanistan, Burma, China, India, Iran, Japan, Kazakhstan, Korea, Nepal, Pakistan, and Russia, whereas only 2 species are found in North America (Chapman and Flux, 1990; Yu et al., 2000; Wilson and Reeder, 2005). Currently, 18 coccidia species (16 *Eimeria,* 2 *Isospora*) are described from all *Ochotona* species. Over 3 summer field seasons (2000–2002), the collared pika, *Ochotona collaris* (Nelson, 1893), and the northern pika, *Ochotona hyperborea* (Pallas, 1811), were collected in Alaska and northeastern Siberia, Russia, respectively, as part of the Beringia Coevolution Project (Hoberg et al., 2003; Cook et al., 2005). The present study was conducted to assess the similarity of coccidia fauna in 2 closely related hosts geographically separated by the Bering Strait.

Pikas were caught with museum snap traps or shot with firearms. Fecal specimens were taken from 88 animals from 6 regional field sites: *O. collaris* were collected from 2 sites in Alaska (N = 53), whereas *O. hyperborea* were collected from 4 sites in northeastern Siberia, Russia (N = 35). The Alaskan sites were Wrangell-St. Elias National Park and Yukon-Charley Rivers National Preserve; 4 regions in northeastern Siberia were sampled, the Omolon, Anadyr, and Kolyma river basins and the Providenya Oblast. Symbiotype host specimens (Frey et al., 1992; Brooks, 1993), in which all oocyst species/forms were seen and identified here, are maintained in the University of Alaska Museum of the North (UAM). Feces were preserved in 2.5% (w/v) aqueous $K_2Cr_2O_7$ solution. Oocysts were isolated, measured, and photographed as described by Duszynski and Wilber (1997).

In all, 25% (22/88) of the samples were positive: 12/35 (34%) *O. hyperborea,* and 10/53 (19%) *O. collaris.* Only 4 pikas were host to multispecies infections of coccidia. Five distinct oocyst morphotypes were observed and these were consistent with previously recognized coccidia species from other pikas. Three coccidia species were recovered from *O. collaris*: *Eimeria calentinei, Eimeria cryptobarretti,* and *Eimeria klondikensis*; 5 were recovered from *O. hyperborea*: *Eimeria banffensis, E. calentinei, E. cryptobarretti, E. klondikensis,* and *Isospora marquardti.* The recovery of *E. cryptobarretti* from *O. collaris* and *O. hyperborea* represents 2 new host records. Previously, *E. cryptobarretti* only had been found in *Ochotona princeps,* the American pika, in Colorado (Duszynski and Brunson, 1973). The recovery of *I. marquardti* from *O. hyperborea* also represents a new host record. The recovery of 3 species of coccidia in Alaskan *O. collaris* represents geographic range extensions, as does the recovery of 5 species from *O. hyperborea* in Siberia. Both hosts were studied by Hobbs and Samuel (1974) from pikas collected in the Yukon Territory, Canada, (*O. collaris*) and Japan (*O. hyperborea*); in 92 *O. collaris* they reported *E. banffensis, Eimeria barretti, Eimeria circumborealis, Eimeria princeps, I. marquardti,* and *Isospora yukonensis,* and in 14 *O. hyperborea* they recovered *E. circumborealis, E. princepsis,* and *Eimeria worleyi.*

Because there have been so few published reports of coccidia from these hosts, we include brief mention of qualitative or quantitative (or both) structures as they differ from the original descriptions, along with taxonomic summaries of the species recovered.

Eimeria banffensis Lepp, Todd, and Samuel, 1973

Type host: O. princeps (Richardson, 1828), American pika.
Other hosts (this study): O. hyperborea.
Type locality: North America: Canada: Alberta, Banff, Jumpingpound, and Sibbald creeks.
Geographic distribution: North America: Canada: Alberta, Banff, Jumpingpound, and Sibbald creeks, 51°N, 115°W; Yukon Territory, Ogilvie Mountains, 64°N, 138°W; U.S.A.: Colorado, Larimer and Clear Creek counties; Asia: Japan: Hokkaido, Daisetzusan National Park; Russia: Siberia, Chukotka, 3 km SSE of confluence of Volchya River and Liman Sea, 64°48′N, 177°33′E (this study).
Prevalence: 5/92 (5%) *O. collaris* (type host) in Yukon Territory; 3/14 (21%) *O. hyperborea* in Japan; 5/35 (14%) *O. hyperborea* in Russia (this study); 40/167 (24%) *O. princeps* in Colorado; 11/145 (8%) *O. princeps* in Alberta.
Material deposited: Skull, skeleton, and tissues of a symbiotype host (this study) are preserved in UAM, as UAM no. 84368 (IF 5252), male, 11 August 2002 (collected by N. E. Dokuchaev, A. A. Tsvetkova). Photosyntype of sporulated oocysts are in the U.S. National Parasite Collection (USNPC) as USNPC no. 87390.
Remarks: The morphology of *E. banffensis* from *O. hyperborea* in Russia is similar to the original description provided by Lepp et al. (1973) for this species collected and described from *O. princeps* in Alberta, Canada. Whereas Duszynski and Brunson (1973) described oocysts that were nearly 2 μm smaller in both length and width, the oocyst sizes of our Russian oocysts did not differ when compared with the original specimens (30 × 25 vs. 30 × 25). Duszynski and Brunson (1973) and Hobbs and Samuel (1974) failed to detect the ~2-μm polar granule that was observed in both this study and the original study by Lepp et al. (1973). The recovery of *E. banffensis* is a new geographic record for this parasite in Russia.

Eimeria calentinei Duszynski and Brunson, 1973

Type host: O. princeps (Richardson, 1828), American pika.
Other hosts (this study): O. collaris, O. hyperborea.
Type locality: North America: Colorado, Larimer County.
Geographic distribution: North America: Canada: Yukon Territory, Ogilvie Mountains, 64°N, 138°W, Alberta, 51°N, 115°W; U.S.A.: Colorado: Clear Creek and Larimer counties; Alaska: Yukon-Charley Rivers National Preserve, NW of Rocky Slope of Mt. Kathryn, S of Woodchopper Creek, 65°12′N, 143°33′W (this study); Asia: Japan: Hokkaido, Daisetzusan National Park; Russia: Siberia, Magadanskaya Oblast, 40 km W Magadan, 59°41′N, 150°20′E (this study).
Prevalence: 5/53 (9%) *O. collaris* in Alaska (this study); 8/92 (9%) *O. collaris* in Yukon Territory; 2/35 (6%) *O. hyperborea* in Siberia (this study); 1/14 (7%) *O. hyperborea* in Japan; 2/111 (2%) *O. princeps* in Alberta; 39/167 (23%) *O. princeps* (type host) in Colorado.
Material deposited: Skin, skull, skeleton, and tissues of 2 symbiotype hosts, one for each host species from this study, are preserved in the UAM: *O. collaris,* UAM no. 58399 (AF 49330), male, 1 August 2001 (collected by H. Henttonen, J. Niemimaa, K. Gamblin, L. B. Barrelli) and *O. hyperborea,* UAM no. 80824 (AF 38535), 4 September 2000 (collected by S. O. MacDonald, N. E. Dokuchaev, K. E. Galbreath). Photosyntype of a sporulated oocyst in the USNPC as no. 87393.
Remarks: The morphology of sporulated oocysts of *E. calentinei* from *O. hyperborea* in Russia and *O. collaris* in Alaska is nearly identical to those described by Duszynski and Brunson (1973) for the same species collected from *O. princeps* in Colorado. The recovery of *E. calentinei* establishes new geographic records for this parasite in Russia and Alaska.

Eimeria cryptobarretti Duszynski and Brunson, 1973

Type host: O. princeps (Richardson, 1828), American pika.
Other hosts (this study): O. collaris, O. hyperborea.
Type locality: North America: U.S.A.: Colorado, Larimer and Clear Creek counties.
Geographic distribution: North America: Colorado: Larimer and Clear Creek counties; Alaska: Wrangell-St. Elias National Park (this study), Yukon-Charley Rivers National Preserve, mountainside NW of Headwater Lake of Crescent Creek, 64°82′N, 143°75′W (this study); Asia: Russia: Siberia, Magadanskaya Oblast, mouth of Kegali River, 64°26′N, 161°47′E (this study).
Prevalence: 6/53 (11%) *O. collaris* in Alaska (this study); 5/35 (14%) *O. hyperborea* in Siberia (this study); 107/167 (64%) *O. princeps* (type host) in Colorado.
Material deposited: Skin, skull, skeleton, and tissues of 2 symbiotype hosts, one for each host species from this study, are preserved in the UAM: *O. collaris,* UAM no. 58213 (AF 49535), 18 July 2001 (collected by H. Henttonen, J. Niemimaa, K. Gamblin, L. B. Barrelli) and *O. hyperborea,* UAM no. 80603 (AF 38233), male, 19 August 2000 (collected by S. O. MacDonald, N. E. Dokuchaev, K. E. Galbreath). Photosyntype and photoparatype of sporulated oocysts are in the USNPC, nos. 87480 and 88170, respectively.
Remarks: The morphology of *E. cryptobarretti* from *O. hyperborea* in Russia and *O. collaris* in Alaska is similar to the description by Duszynski and Brunson (1973) for the same species collected from *O.*

TABLE I. Ten studies documenting the presence of coccidia (*Eimeria*, *Isospora* spp.) in pikas (*Ochotona* spp.) from 2 continents.

	North America		Asia			
Coccidia species	*O. collaris*	*O. princeps*	*O. dauurica*	*O. hyperborea*	*O. pallasi*	*O. rufescens*
E. balchanica	—	—	—	—	—	4*
E. banffensis	5	1, 3, 5, 7	—	5, 10	—	—
E. barretti	5	5, 6	—	—	—	—
E. calentinei	5, 10	1, 3, 5	—	5, 10	—	—
E. circumborealis	5	—	—	5	—	—
E. cryptobarretti	10	1, 3	—	10	—	—
E. daurica	—	—	8	—	—	—
E. erschovi	—	—	8	—	9	—
E. klondikensis	5, 10	1, 5	—	5, 10	—	—
E. metelkini	—	—	8	—	—	—
E. ochotona	—	—	8	—	—	—
E. pallasi	—	—	—	—	6	—
E. princeps	5	1, 3, 5	—	5	—	—
E. shubini	—	—	—	—	6	—
E. worleyi	—	1, 6	—	5	—	—
E. sp.	—	—	—	—	6	—
I. marquardti	5	1, 2, 5	—	10	—	—
I. yukonensis	5	—	—	—	—	—

* 1. Duszynski, 1974; 2. Duszynski and Brunson, 1972; 3. Duszynski and Brunson, 1973; 4. Glebezdin, 1978; 5. Hobbs and Samuel, 1974; 6. Lepp et al., 1972; 7. Lepp et al., 1973; 8. Machulsky, 1949; 9. Svanbaev, 1958; 10. Present study.

princeps in Colorado, U.S.A. The recovery of *E. cryptobarretti* establishes new host and geographic records for this parasite in Russia and Alaska, U.S.A. The authors of the original description hesitated to state if there was a micropyle on the oocyst, but we now believe that *E. cryptobarretti* does indeed have one.

Eimeria klondikensis Hobbs and Samuel, 1974

Type host: O. collaris (Nelson, 1893), collared pika.

Other hosts (this study): O. hyperborea.

Type locality: North America: Canada: Yukon Territory, Ogilvie Mountains, 64°N, 138°W.

Geographic distribution: North America: Canada: Yukon Territory, Ogilvie Mountains, 64°N, 138°W; Alberta, 51°N, 115°W; U.S.A.: Colorado: Clear Creek County; Alaska: Wrangell-St. Elias National Park and Preserve, SE of Rock Lake, 21 July 2001, 61°47′N, 141°12′W (this study); Yukon-Charley Rivers National Preserve (this study); Asia: Japan: Hokkaido, Daisetzusan National Park; Russia: Siberia, Chukotka, 3 km SSE of confluence of Volchya River and Liman Sea, 64°48′N, 177°33′E (this study).

Prevalence: 2/53 (4%) *O. collaris* in Alaska (this study); 3/92 (3%) *O. collaris* (type host) in Yukon Territory; 1/35 (3%) *O. hyperborea* in Siberia (this study); 2/14 (14%) *O. hyperborea* in Japan; 7/111 (6%) *O. princeps* in Alberta; 62/224 (28%) *O. princeps* in Colorado.

Material deposited: Skin, skull, skeleton, and tissues of 2 symbiotype hosts, one for each host species from this study, are preserved in the UAM: *O. collaris,* UAM no. 56067 (AF 54551), female, 21 July 2001 (collected by S. Kutz, A. Tsvetkova, A. A. Eddingaas, M. McCain) and *O. hyperborea,* UAM no. 84369 (IF 5253), male, 11 August 2002 (collected by N. E. Dokuchaev, A. A. Tsvetkova). We deposited a photoneotype of a sporulated oocyst in the USNPC as no. 99671, because no previous authors had archived a type specimen of this parasite.

Remarks: The morphology of *E. klondikensis* from *O. collaris* in Alaska and *O. hyperborea* in Russia is similar to the description provided by Hobbs and Samuel (1974) for the same species collected and described from the same hosts in Canada and Japan, respectively. The recovery of *E. klondikensis* establishes new geographic records for this parasite in Russia and Alaska, U.S.A. Both a line drawing and a photomicrograph of the sporulated oocyst of this species appeared in the original description.

Isospora marquardti Duszynski and Brunson, 1972

Type host: O. princeps (Richardson, 1828), American pika.

Other hosts (this study): O. hyperborea.

Type locality: North America: U.S.A.: Colorado, Ft. Collins, Clear Creek, and Larimer counties.

Geographic distribution: North America: U.S.A.: Colorado, Ft. Collins, Clear Creek, and Larimer counties; Canada: Yukon Territory, Ogilvie Mountains, 64°N, 138°W; Alberta, 51°N, 115°W; Asia: Russia: Siberia, Chukotka, Ulhum River, 15 km W of Chaplino Village, 64°25′N, 172°32′E (this study).

Prevalence: 1/92 (1%) *O. collaris* in the Yukon Territory (this study); 1/35 (3%) *O. hyperborea* in Siberia (this study); 1/111 (<1%) *O. princeps* in Alberta; 25/167 (15%) *O. princeps* (type host) in Colorado.

Material deposited: Skull, skeleton, and tissues of a symbiotype host from this study are preserved in the UAM as UAM no. 83836 (IF 7569), female, 28 July 2002 (collected by V. F. Fedorov, K. E. Galbreath). Photosyntype of a sporulated oocyst is in the USNPC as no. 87408.

Remarks: The morphology of sporulated oocysts of *I. marquardti* from *O. hyperborea* in Russia differ slightly from those of Duszynski and Brunson (1972) collected and described from *O. princeps* in Colorado; the latter had oocysts and sporocysts that were larger in both length and width (31 × 30 and 19 × 12 vs. 28 × 27 and 17 × 11) than those of our Russian specimens. Still, both oocysts and sporocysts reported here were larger than those measured by Hobbs and Samuel (1974) from *O. collaris* (23 × 22 and 15 × 9). Oocysts of some species are known to exhibit phenotypic plasticity (see Duszynski et al., 1992) and, given the similarity of qualitative data, we believe these oocysts are *I. marquardti.* The recovery of *I. marquardti* establishes a new host and geographic record for this parasite in Russia.

Machulsky (1949) published the first paper on coccidia in pikas. Since then, 9 additional papers, including this one, have described a total of 18 coccidia species in 6 *Ochotona* species: 2 from North American and 4 from Asia (Table I). The coccidia reported from 3 of those 6 hosts, *O. collaris, O. princeps,* and *O. hyperborea,* which are the best studied hosts (Table I), are remarkably similar. These hosts have all been studied on multiple occasions and 10 coccidia have been reported from them. Six of 10 (60%) coccidia have been reported from all 3 hosts, whereas 3 others have been reported from at least 2 hosts. One species, *Isospora yukonensis,* has been reported from only a single individual of *O. collaris* (Hobbs and Samuel, 1974). The overlap of coc-

cidia species among *O. collaris, O. hyperborea,* and *O. princeps* suggests the possibility that these coccidia may have evolved from a common ancestor, i.e., that shared coccidia faunas in 3 closely related pika species may reflect a single origin for the parasites in their common ancestor. On the other hand, this pattern may indicate that each coccidium had a common ancestor in the ancestor of the pikas. Thus, the parasite community may have a recent origin, but this doesn't say anything about relationships among these coccidia.

Except for *Eimeria erschovi* Machulsky, 1949, the 7 coccidia identified from the remaining Asian pikas, *Ochotona dauurica* (*Eimeria daurica, Eimeria metelkini, Eimeria ochotona,* in 1949), *Ochotona pallasi* (*Eimeria pallasi, Eimeria shubini, Eimeria* sp., in 1958), and *Ochotona rufescens* (*Eimeria balchanica,* in 1978), only have been identified once, each from 2–3 specimens of their single host species. Initially, the lack of overlap indicates that these coccidia may be more host-species specific, but nothing substantive is known about host specificity in pika coccidia. On the other hand, given the known distributions of the 3 host species, it is unlikely that these coccidia would ever come into contact with an *Ochotona* species different from the one in which it was first described; *O. rufescens,* the Afghan pika, is geographically separated from the other 2 species and, although the ranges of *O. dauurica* and *O. pallasi* share some overlap, e.g., Mongolia, these species are separated both by altitude and biome (high mountain vs. desert, respectively). In addition, the obvious sampling bias doesn't allow meaningful comparisons. Finally, the question must be asked whether any of these 7 coccidia even still exist since 2 of the 3 host species are either endangered (*O. pallasi*) or threatened (*O. rufescens*).

Our a posteriori hypothesis was that the similarity, or disparity, of coccidia infecting pikas would reflect the systematics and phylogenetics of the hosts. Work by Yu et al. (2000) on the phylogeny of 19 pika species included *O. hyperborea, O. princeps, O. pallasi,* and *O. dauurica*; sequences from *O. collaris* and *O. rufescens* were not incorporated. The data of Yu et al. (2000) indicated that there are 3 pika clades: a shrub-steppe group of 7 species (including *O. dauurica*), a northern group of 5 species (including *O. hyperborea, O. pallasi,* and *O. princeps*), and a mountain group of 7 species. Host–parasite data to date (Table I) support the notion that *O. hyperborea* and *O. princeps* may be infected by the same coccidia because they have descended from a recent common ancestor. If true, this would predict that the same or similar coccidia species will be found in other species from the northern group of Yu et al. (2000). In other words, the morphological similarity of the coccidia in this study might reflect close phylogenetic relationships that are a consequence of the close relationship between the hosts.

The data of Yu et al. (2000) posit that *O. princeps* is the most basal member of the northern group. Interestingly, *O. pallasi* is infected by an entirely different set of coccidia from other hosts in the northern clade. In fact, *O. pallasi* is infected by *E. erschovi,* a coccidium first identified from *O. dauurica,* a member of the shrub-steppe group of pikas. Despite an older association between *O. dauurica* and *O. pallasi,* it is possible that *E. erschovi* is a generalist parasite capable of a broad co-accommodation of hosts (Brooks, 1979). In other words, the association between *E. erschovi* and 2 deeply divergent pika lineages may suggest the generalist nature of this coccidium. These hosts are in relatively close contact as the range of *O. pallasi* overlaps that of *O. dauurica,* although they occupy different habitats (Chapman and Flux, 1990); unfortunately, it is not known if any burrowing or talus-dwelling pikas live in enough proximity to connect these pika lineages. Perhaps the other *Eimeria* spp. identified from *O. pallasi* (*Eimeria pallasi, Eimeria shubini,* and *Eimeria* sp.) are more derived (than those infecting *O. princeps*) and are results of recent speciation. This would keep the cospeciation hypothesis alive, but it is also possible that a host switch could have led to this association. Only phylogenetic (sequence) data for these coccidia will resolve the relationships among them.

It also is recognized that the dichotomy seen in Table I, where 3 hosts overlap in eimeriid fauna and the other 3 hosts have divergent fauna, could be the result of poor species descriptions. Both Hobbs and Samuel (1974) and Lepp et al. (1972) addressed this possibility. Hobbs and Samuel (1974) noted the extreme similarity between many of the ''continental Asian'' and the ''North American'' coccidia. In all cases, there were enough differences among those coccidia to prevent the synonymy of species. Before conclusions can be made regarding the validity of species descriptions, more Asian pikas must be surveyed. Finding evidence for cryptic species of coccidia in pikas also could be an important issue for sorting out the origins of their host–parasite associations.

In conclusion, we emphasize 3 major points: (1) the similarity in coccidia fauna among *O. princeps, O. collaris,* and *O. hyperborea*; (2) the different and more diverse coccidia parasites in Asian hosts; and (3) the apparent widespread species of coccidia found in pikas representing 2 different host clades.

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A *Ribeiroia* Spp. (Class: Trematoda)—Specific PCR-Based Diagnostic

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ABSTRACT: Increased reporting of amphibian malformations in North America has been noted with concern in light of reports that amphibian numbers and species are declining worldwide. *Ribeiroia ondatrae* has been shown to cause a variety of types of malformations in amphibians. However, little is known about the prevalence of *R. ondatrae* in North America. To aid in conducting field studies of *Ribeiroia* spp., we have developed a polymerase chain reaction (PCR)-based diagnostic. Herein, we describe the development of an accurate, rapid, simple, and cost-effective diagnostic for detection of *Ribeiroia* spp. infection in snails (*Planorbella trivolvis*). Candidate oligonucleotide primers for PCR were designed via DNA sequence analyses of multiple ribosomal internal transcribed spacer-2 regions from *Ribeiroia* spp. and *Echinostoma* spp. Comparison of consensus sequences determined from both genera identified areas of sequence potentially unique to *Ribeiroia* spp. The PCR reliably produced a diagnostic 290-base pair (bp) product in the presence of a wide concentration range of snail or frog DNA. Sensitivity was examined with DNA extracted from single *R. ondatrae* cercaria. The single-tube PCR could routinely detect less than 1 cercariae equivalent, because DNA isolated from a single cercaria could be diluted at least 1:50 and still yield a positive result via gel electrophoresis. An even more sensitive nested PCR also was developed that routinely detected 100 fg of the 290-bp fragment. The assay did not detect furcocercous cercariae of certain Schistosomatidae, *Echinostoma* sp., or *Sphaeridiotrema globulus* nor adults of *Clinostomum* sp. or *Cyathocotyle bushiensis*. Field testing of 137 *P. trivolvis* identified 3 positives with no overt environmental cross-reactivity, and results concurred with microscopic examinations in all cases.

Concern over declining numbers and species of amphibians has come to the forefront over the past 20 yr (Barinaga, 1990; Blaustein and Wake, 1990; Phillips, 1990; Pechmann et al., 1991; Wake, 1998). Suggested factors, singly or in synergism, that have been hypothesized as reasons for the decline of this class of animals include habitat destruction (Kolozsvary and Swihart, 1999; Houlahan and Findlay, 2003), UV irradiation (Blaustein et al., 1998, 2003), introduced species (Knapp and Mathews, 2000), climate change (Beebee, 1995; Corn, 2005), and various pathogens (Daszak et al., 2003). A current review of the factors is found in Beebee and Griffiths (2005). Amphibian malformations are of growing concern, because they have been observed with increased prevalence in North America (Ouellet, 2000). Although malformations have the potential to deleteriously affect populations or species at particular sites, they have not been empirically linked to global or regional declines. Recent reports (Johnson et al., 1999, 2002; Lannoo et al., 2003; Schoff et al., 2003; Schotthoefer et al., 2003) have implicated the trematode *Ribeiroia ondatrae* as a causative agent of some types of malformations. Little is known about the distribution of this parasite in its hosts within North America. Wilson et al. (2005) identified 3 species of *Ribeiroia*: *R. ondatrae* within the Americas; *R. marini* in the Carribbean, and *Cercaria lilet*a in Africa. *Ribeiroia ondatrae* has a 3-host life cycle with 2 aquatic intermediate hosts and a predator definitive host, usually a bird or mammal. *Planorbella* spp. serves as first intermediate host, with fish and various amphibians as second intermediate hosts. Exogenous factors, which include pesticides (Kiesecker, 2002), and eutrophication, which leads to a dominance of *Planorbella* spp. (Johnson and Chase, 2004), have been shown to increase malformation rates. Currently, *Ribeiroia* spp. infections in the first intermediate host are diagnosed by dissection of live or freshly dead snail hosts for various larval stages, which requires training and substantial time to locate infected tissues to identify the parasite correctly. Identifying larvae early in development after miracidial penetration but before the development of the cercariae is difficult, if not impossible, using morphological characters. To increase the speed and accuracy in the examination of large numbers of snails for the presence of *Ribeiroia* spp., to reduce labor costs, and to simplify training required, we have developed a genus-specific polymerase chain reaction (PCR)-based diagnostic that targets the second internal transcribed spacer (ITS-2) region of the ribosomal RNA gene cluster (Morgan and Blair, 1998; Kostadinova et al., 2003; Wilson et al., 2005). By using various combinations of 4 oligonucleo-

TABLE I. Oligonucleotides and PCR profiles used to detect *Ribeiroia* sp.

Primer	Sequence	T_m	% GC	Use/product size
21-up	AGTCATGGTGAGGTGCAGTGA	59.7	52.4	with 18-dn, 290 bp, profile 1
18-dn	AGACCGCTTAGATAGCAG	51.4	50.0	with 21-up, 290 bp, profile 1
18-up	CGTGTTTGGCGATTTAGT	51.4	44.4	Nested reaction with 19-dn, 164 bp, profile 2
19-dn	TCAAAAATGAAGCAACAGT	49.1	31.6	Nested reaction with 18-up, 164 bp, profile 2

Profile 1

1X	94 C	4 min						
	94 C	15 sec		94 C	15 sec			
10X	59 C	30 sec	26X	59 C	30 sec	1X	72 C	7 min
	72 C	45 sec		72 C	90 sec		4 C	Hold

Profile 2. As above except anneal at 53 C versus 59 C.

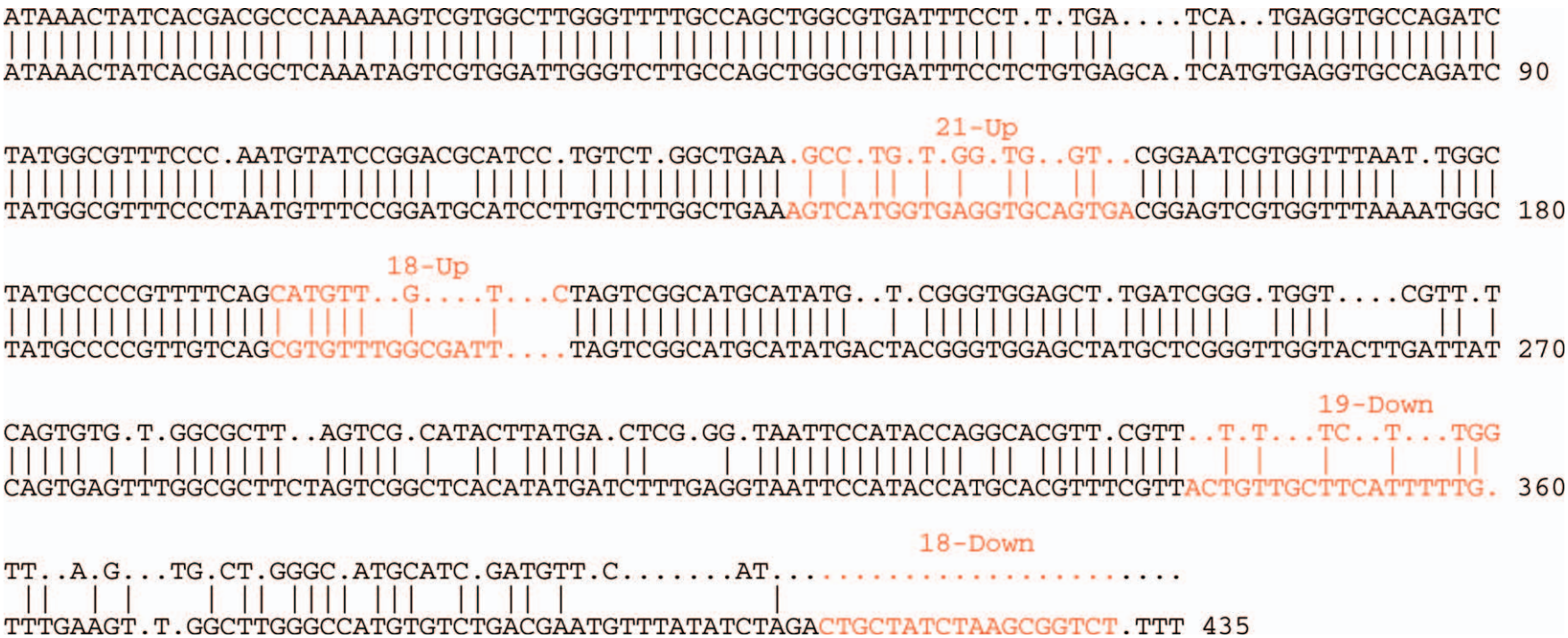

FIGURE 1. Comparison of ITS-2 consensus sequences generated with the program Gap shows only invariant positions within each genus (oligonucleotide primers are shown in red, 18 and 19 down are shown as reverse complements). The *Ribeiroia* sp. consensus sequence (bottom line) was derived from 9 isolates of the parasite (AY761142, AY761147, U58102, U58100, U58098, U58097, AY168931, AF026791, and AF067851), whereas the *Echinostoma* sp. sequence (top line) was from 7 database files (U58102, U58100, U58098, U58097, AY168931, AF026791, and AF067851) using the program Pretty (gap weight 5; gap length weight 1).

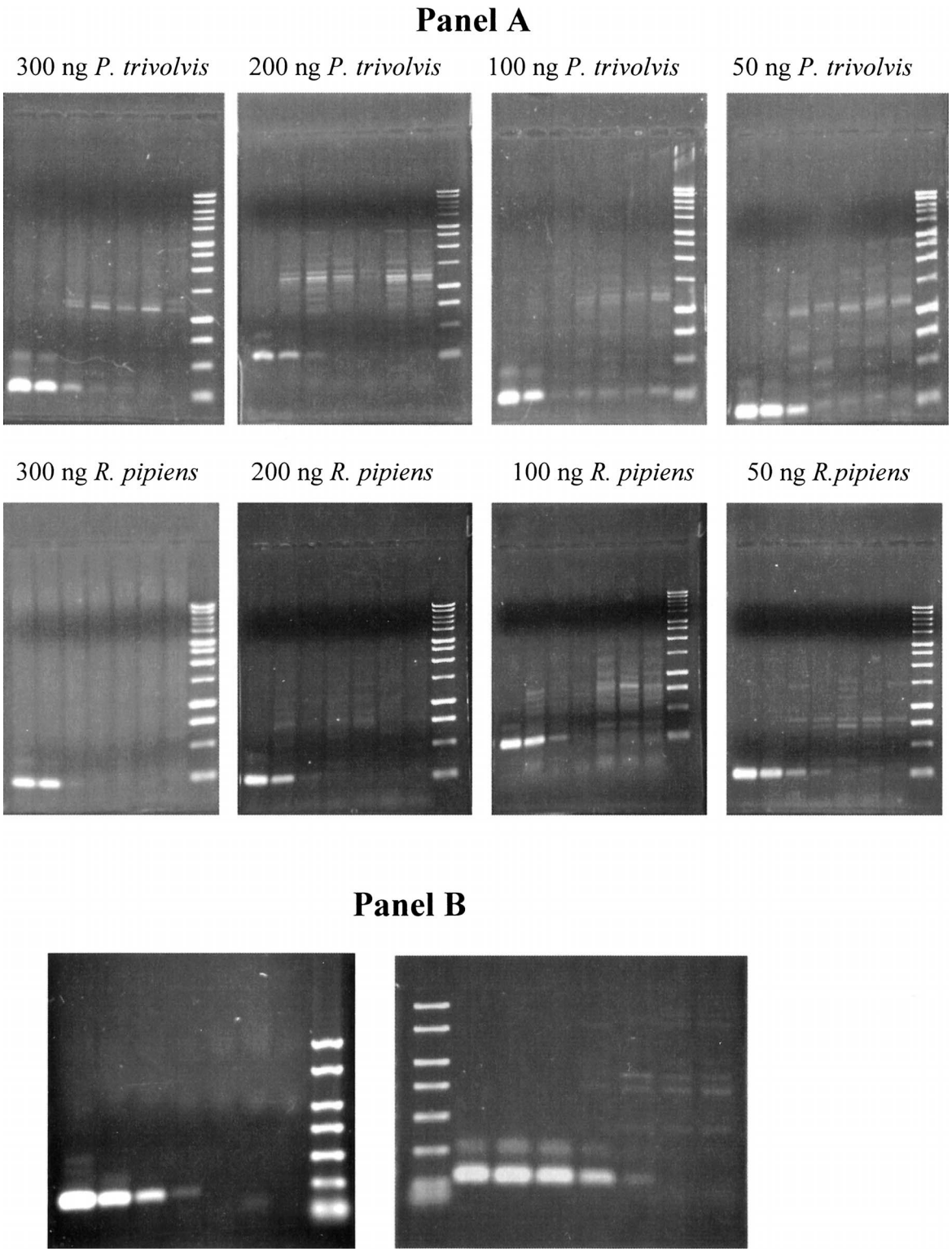

FIGURE 2. (**A**) Production of the *Ribeiroia*-specific 290-bp DNA (lowest band) in the presence of *Planorbella trivolvis* (row 1) or *Rana pipiens* (row 2) DNA. Two nanograms of *Ribeiroia* sp. cercarial DNA (lane 1) was diluted 10-fold in the presence of 300, 200, 100, or 50 ng of DNA (left to right) from *P. trivolvis* (row 1) or from *R. pipiens* (row 2). Routine detection was 0.2 ng (lane 2, all gels); and optimally, 20 pg of DNA could be detected (top row, lane 3, 50 ng of *P. trivolvis* DNA). The five lowest standards are 1,500, 1,000, 750, 500, and 250 bp. (**B**) Sensitivity of ITS-2 (L) and nested (R) *Ribeiroia* sp.-specific PCR. Left gel shows PCR results with DNA from 20 cercariae (lane 1) and 10-fold dilutions thereof (lanes 2–6; lane 7, no DNA) with primers 21-up/18-dn and subjected to profile 1 (290-bp target). DNA from less than 1 cercaria was easily detectable (lanes 2 and 3) with little or no extraneous DNAs produced. Right gel shows results from the second nested reaction, which used a purified 290-bp template from the first reaction and primers 18-up and 19-dn to produce a 164 bp DNA. Ten-fold dilutions of the 290-bp fragment (lanes 2–7) ranged from 100 pg to 1 fg; lane 8 shows a reaction with no template DNA. One hundred femtograms (<1 genome equivalent) of the 290-bp fragment could be routinely detected (lane 5). Standards in both gels are 2,000, 1,500, 1,000, 750, 500, 300, 150, and 50 bp.

tide primers, the assay can be performed as a single-tube PCR or as a nested reaction for greater sensitivity. Table I summarizes primer characteristics, uses, and profiles. Desirable assay characteristics include sufficient sensitivity to detect early infection of snails, accuracy, cost-effectiveness, simplicity, and the ability to detect parasite DNA in the presence of excess frog or snail host DNA. In this article, we examined how the primary pair of primers worked to amplify target sequences in the presence of variable amounts of frog or snail DNA with regard to sensitivity and specificity.

ITS-2 sequences unique to *Ribeiroia* spp. were tentatively identified by a 2-step process (Fig. 1): first, by producing an ITS-2 consensus sequence from both *Ribeiroia* and *Echinostoma* spp. with the program Pretty (Accelrys, San Diego, California); and second, by an alignment of the 2 consensus sequences with the program Gap (Accelrys) to identify areas of divergence. *Echinostoma* spp. were selected for comparison because sequence data for more closely related organisms were not available. From sequences potentially unique to *R. ondatrae*, we designed 4 oligonucleotide primers, denoted 21-up, 18-dn, 18-up, and 19-dn (Table I) for use as PCR primers. Primer dimer formation was not predicted to be problematic by the programs Gap and Bestfit (Accelrys), and the theoretical melting temperatures (T_m) provided by the manufacturer (Integrated DNA Technologies, Inc., Coralville, Iowa). Melting point considerations and degree of mismatch with *Echinostoma* spp. sequences lead us to first test primers 21-up with 18-dn (single reaction) and 18-up with 19-down (nested reaction), which produced DNA fragments of 290 base pairs (bp) and 164 bp, respectively.

PCR mixtures contained 200 μM each deoxynucleoside-5[prime]-triphosphate, 300 nM 18-up, 300 nM 18-down, 2.6 U of Expand High Fidelity Enzyme (Roche Diagnostics, Indianapolis, Indiana), and 1.5 mM $MgCl_2$ in 50 μl. Alternatively, 25-μl reactions with PCR Beads (G. E. Healthcare, Buckinhamshire, U.K.) with primer and DNA concentrations, as described above, produced identical results. After amplification, samples were analyzed by agarose gel electrophoresis for a DNA fragment of specific size (290 bp or 164 bp). The identity of the amplified DNA was confirmed to be *Ribeiroia* spp. by sequence analysis. Amplified DNA was purified directly from a 50-μl PCR reaction using Wizard PCR Preps (Promega, Madison, Wisconsin), mixed with the PCR primer (10 ng of DNA and 10 of pmol primer) in water and sent to Northwoods DNA Inc. (Solway, Minnesota). The sequence produced was compared with the *R. ondatrae* sequence using the program BestFit (Accelrys).

Ribeiroia ondatrae genomic DNA was purified from cercariae shed by *Planorbella trivolvis*, using a DNeasy kit (cultured cells protocol; QIAGEN, Valencia, California). Specificity of the assay with primers 21-up and 18-dn was examined in the presence of excess DNA isolated from hosts *P. trivolvis* and *Rana pipiens* (DNeasy, animal tissue protocol; QIAGEN). Figure 2A shows the detection of a serial 10-fold dilution of 2 ng of *R. ondatrae* cercarial DNA (lane 1, with 21-up/18-dn) in the presence of 300, 200, 100, and 50 ng of *P. trivolvis* or *R. pipiens* DNA. Neither of these excess DNAs was problematic, although *P. trivolvis* DNA did produce slightly more nonspecific amplification products than did *R. pipiens* DNA.

In the presence of excess *P. trivolvis* or *R. pipiens* DNA, the *Ribeiroia*-specific 290-bp fragment was consistently produced (verified by sequencing bands excised from such gels). Row 1 of Figure 2A shows production of the diagnostic 290-bp fragment in the presence of 4 levels of *P. trivolvis* DNA (300, 200, 100, and 50 ng/50 μl). No intense DNA fragments of similar size that might obscure detection of the diagnostic 290-bp fragment were produced at any of the levels of host DNA tested. Some low-level nonspecific DNAs were produced (e.g., 300 ng, lanes 3–6) that were distinctly different sizes (≈1,200 bp) from the 290-bp DNA from *R. ondatrae* and are not seen as an impediment to correct assay interpretation.

Clearly, faint 290-bp bands, such as those observed in lanes 4–7 in most of Figure 2A, should be considered negative results. In the absence of *P. trivolvis* or *R. pipiens* DNA, these nonspecific bands were not present, so they probably represent weakly amplified host DNA sequences that in essence define the negative background signal that must be significantly exceeded for a positive determination. In addition, when field testing either *P. trivolvis* or *R. pipiens* tissue, total DNA added did not exceed 100 ng, a level where interpretation of the agarose gel results is straightforward. Overall, many of the gel lanes in Figure 2A contained only the specific fragment.

The single-tube PCR with primers 21-up and 18-dn can routinely detect less than a single cercariae equivalent (CE) of *R. ondatrae* DNA. Figure 2B shows the results of serial 10-fold dilutions of this target DNA starting with 20 CE. A positive reaction was clearly and consistently seen with 0.2 CE (lane 3). The amount of input target was determined by extracting DNA from 20 cercariae (with carrier RNA, 10 μg/sample) collected from *R. ondatrae*-infected *P. trivolvis*. Because this procedure assumed a 100% recovery, the assay can probably detect less than 0.2 CE. This also was convincingly demonstrated using DNA isolated from a single cercaria (with carrier RNA, 10 μg/sample) with identical results (data not shown). Furthermore, if an aliquot of the primary PCR reaction was subjected to a second round of PCR with nested primers 18-up and 19-dn, as little as 100 fg (<1 genome equivalent) of the 290-bp template could be detected (Fig. 2B). Although the conventional PCR assay (21-up/18-dn) has not been extensively field tested, many co-occurring and potentially cross-reactive organisms, including *Echinostoma* sp., have been examined.

Two field samples of *P. trivolvis* infected with *Echinostoma* sp. rediae and cercariae were tested by excising parasite tissue for DNA extraction. The 2 samples, at 3 different levels of target DNA (10-fold dilutions), produced no DNAs in the 290-bp size range and little nonspecific DNA of any size. Such samples, when spiked with *R. ondatrae* target DNA (100 ng), produced a 290-bp band, which indicated that lack of *Echinostoma* sp. reactivity was not due to inhibitory substances present in these samples. And, an independent examination of trematodes that included *Clinostomum* sp., *Cyathocotyle bushiensis*, or *Sphaeridiotrema globulus*, showed no cross-reactivity in amplifications with 21-up/18-dn. *Epistylis* sp. (class Ciliata), an epibiont from snail shells, did give a positive reaction that sequencing revealed to be due to filter feeding upon *R. ondatrae* sp. cercariae. The assay does not detect xiphidiocercariae from *P. trivolvis*; and as expected, the assay does strongly detect *R. marini* cercariae.

Snails from an ongoing field study in Illinois were first microscopically examined for *R. ondatrae* cercariae. The hepatopancreas was then excised and pooled (1–3 individuals) into 137 tissue samples from which DNA was purified (DNeasy kit; QIAGEN), and samples were assayed via PCR with 21-up/18-dn. No cross-reactivity or other interference was observed, and the assay identified 3 positive pools of tissue, which corroborated microscopic examinations. Although several groups of tissue were from snails where infections were very immature (laboratory reared snails were placed in field cages for 3–4 wk), and identification based on morphological characters was inconclusive, the PCR assay enabled positive identification of *R. ondatrae*. Other more limited field tests also gave results that correlated well with microscopic examinations as 9 *P. trivolvis* from Duck Lake, Minnesota, and 3 *P. trivolvis* from Merriville, Minnesota, yielded 6 positives, also visible microscopically in all cases. Overall, the assay is very robust, with no known cross-reactive DNA from environmental sources that interferes with its interpretation. Although the assay does require some subjectivity to discern weak positive signals from background negatives, it is more rapid, more sensitive, and far simpler to train new personnel to conduct these procedures than to develop the ability to microscopically identify the different life cycle stages of *Ribeiroia* spp. and other organisms that may be present within snails. This assay will be useful in studies where a large number snails must be examined for *Ribeiroia* spp. infections, and it will help determine where and when *Ribeiroia* spp. infections can have a deleterious impact on frog populations.

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Human Neurocysticercosis: Rightward Hemisphere Asymmetry in the Cerebral Distribution of a Single Cysticercus

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ABSTRACT: The distribution of single cysticerci between cerebral hemispheres was studied in 227 adult cases of calcified and vesicular neurocysticercosis (NC). A rightward lateralization of calcified cysticerci was significant only in women, whereas vesicular cysticerci were equally distributed in both hemispheres. Factors related with the differences in the inflammatory response and in the regional cerebral blood flow between genders could be involved.

Human neurocysticercosis (NC) is a severe and frequent neurological disease in developing countries (Sciutto et al., 2000; Garcia et al., 2003; Fleury et al., 2006), and it is considered as a re-emergent disease in developed ones due to the increase of immigration (DeGiorgio et al., 2005). NC is caused by the larval stage of the cestode parasite *Taenia solium* (cysticercus), which may develop in the central nervous system of its human host. Although cerebral lateralization is well recognized in several brain physiological functions, and the brain is progressively recognized as capable of modulating the local and systemic immune response (Tarkowski et al., 1995; Fu et al., 2003; Meador et al., 2004), a possible asymmetric distribution of cysticerci or of any other brain parasites, i.e., toxoplasmosis, malaria, has not been investigated. The present study examines the hemisphere distribution of single cysticerci in the human brain.

In total, 227 human adult NC cases with a single cysticercus were included. The anatomic status of the parasites (189 calcified, 38 vesicular), their hemispherical location (132 right, 95 left), and the diagnostic tool used (radiology in 172 and autopsy in 55) were recorded. Ninety-four men and 133 women were included. All the patients with radiological diagnosis had a CT scan. In a subgroup of them (50 cases), magnetic resonance imaging (MRI) was also used. In all of these 50 cases, CT scan and MRI results were consistent. Only cases with a single lesion closely resembling those described for *T. solium* cysticerci

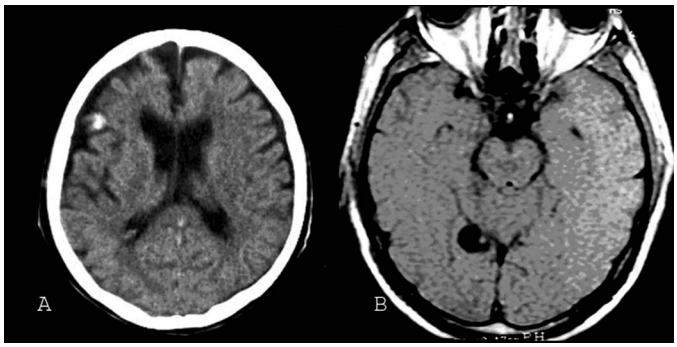

FIGURE 1. (**A**) CT scan. Single calcified cysticerci located in right hemisphere. Small hyper dense nodules in CT (<2 mm) were considered as calcified cysticerci because of showing the typical radiological features of calcified cysticerci and considering the high NC prevalence in Mexico. (**B**) MRI. Single vesicular NC located in the right hemisphere.

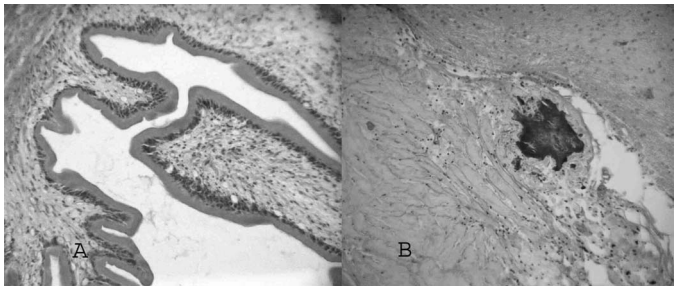

FIGURE 2. (**A**) Microscopic identification of live cysticerci is based on the identification of the 3-layered structure containing fluid. (**B**) The microscopic diagnosis of calcified cysticerci must rest on clinical and epidemiological data because specific structures are absent. Other possible sources of calcified nodules in the brain, such as old tuberculomas or meningiomas should be excluded, because the best morphologic diagnosis of such lesions is of consistency with calcified cysticerci.

(Rodriguez-Carbajal and Boleaga-Duran, 1983; Villagran and Olvera, 1988) were included. The inclusion criteria were: reliable diagnosis by CT scan and/or MRI (Fig. 1), or macro- and microscopic findings at autopsy (Fig. 2).

Distribution of single cysticerci in the cerebral hemispheres (right, left) was evaluated with respect to stage of the parasite (calcified, vesicular), sex (women, men), and method of diagnosis (CT scan or necropsy). Results were analyzed with the SPSS software. Significant differences in the frequency of parasites in each hemisphere were evaluated using a nonparametric χ^2 test.

Table I shows that there is a right-side localization of calcified cysticerci in the brain hemisphere ($P = 0.002$), which was statistically significant in CT scan and autopsy results in women only ($P = 0.03$, and $P = 0.003$, respectively). In contrast with calcified cysticerci, no statistically significant lateralization of vesicular cysticerci was found in either women or men using either the CT scan, or in autopsy cases.

Evidence is presented of a statistically significant preferential bias towards the right hemisphere of single calcified cysticerci in women. Vesicular cysticerci did not show a hemisphere preference. Right-side bias of single calcified cysticerci may result from the more frequent entry of the parasite into the right than into the left hemisphere or from a differential management of the host–parasite relationship between hemispheres. The fact that vesicular parasites locate equally in the right and left hemisphere suggests equal entry of parasites into both hemispheres and emphasizes the differential management hypothesis. It is possible that the right hemisphere may calcify incoming parasites more effectively, or the left hemisphere might destroy them more effectively without sequel, or both. Because it is only observed in women, this bias could be promoted by increased inflammatory response observed in NC females and its particular hormonal local environment (Fleury et al., 2004; Chavarria et al., 2005; Soucy et al., 2005). Difference in regional cerebral blood flow (rCBF) between women and men recently reported (Van Laere et al., 2001; Pirson et al., 2006) could be involved. In this respect, it was reported that rCBF was increased in the right sensorimotor cortex and decreased in the left temporal cortex in women. It is possible that a higher rCBF in right hemisphere in women promotes a higher cysticerci calcification rate due to the arrival of a significant number of immunocompetent cells. At present, there is no way of discerning which immune mechanism involved in the host–parasite relationship would be responsible for this symmetry bias of females. Further research is necessary to evaluate lateralization in the natural course of other immunoinflammatory confrontations in the brain and to understand the reasons of such an observation.

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TABLE I. Distribution of single calcified and vesicular cysticerci in 227 human NC cases.

	Calcified				Vesicular			
Diagnosis	Total	Right	Left	*P**	Total	Right	Left	*P*
Radiological								
Men	48	28	20	0.25	21	10	11	0.83
Women	88	54	34	0.03	15	4	11	0.07
Histopathological								
Men	23	11	12	0.8	2	2	0	—
Women	30	23	7	0.003	0	0	0	—
Totals	189	116	73	0.002	38	16	22	0.33

* *P*: *P* value of the difference between right/left parasite localization in each cases group.

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Ribeiroia ondatrae Cercariae Are Consumed by Aquatic Invertebrate Predators

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ABSTRACT: Trematodes amplify asexually in their snail intermediate hosts, resulting in the potential release of hundreds to thousands of free-living cercariae per day for the life of the snail. The high number of cercariae released into the environment undoubtedly increases the probability of transmission. Although many individual cercariae successfully infect another host in their life cycle, most fail. Factors that prevent successful transmission of cercariae are poorly understood. Microcrustaceans and fish have been observed to eat cercariae of some species, although the possibility that predation represents a significant source of mortality for cercariae has been largely unexplored. We tested the cercariophagic activity of several freshwater invertebrates on *Ribeiroia ondatrae*, a trematode that causes limb deformities in amphibians. Individuals of potential predators were placed into wells of multiwell plates with 10–15 cercariae, and numbers of cercariae remaining over time were recorded and compared with numbers in control wells that contained no predators. Of the species tested, *Hydra* sp., damselfly (Odonata, Coenagrionidae) larvae, dragonfly (Odonata, Libellulidae), larvae, and copepods (Cyclopoida) consumed cercariae. In some cases, 80–90% of the cercariae offered to damselfly and dragonfly larvae were consumed within 10 min. In most cases, predators continued to consume cercariae at the same average rates when offered cercariae together with individuals of an alternate prey item. *Hydra* sp. ate fewer cercariae in these trials. Our findings suggest the need for field and laboratory studies to further explore the effects of predators on transmission of *R. ondatrae* to amphibian larvae. In addition, the results suggest that conservation of the biodiversity and numbers of aquatic predators may limit adverse impacts of trematode infections in vertebrate hosts.

Coinciding with declines in North American amphibian populations over the past decade is an increase in the frequency of limb deformities, with 50% or more deformities occurring in many populations (Johnson et al., 2002, 2003). Although it has yet to be determined if some of the deformities observed in wild populations can be attributed to other teratogenic factors, such as UV radiation and pesticide contamination, laboratory and field-based investigations have pointed to infection by trematode cercariae of the species *R. ondatrae* as the most likely cause, at least of supernumerary and branched limbs (Johnson et al., 1999, 2002; Blaustein and Johnson, 2003; Taylor et al., 2006).

Species of *Ribeiroia* have complex life cycles in which snails become infected by miracidia and release free-swimming cercariae in large numbers (A. Schotthoefer, pers. obs.). The cercariae go on to infect tadpoles or fish, encysting as metacercariae. When these latter infected intermediate hosts are ingested by avian or mammalian definitive hosts, *Ribeiroia* spp. develop to their adult stage and may engage in sexual reproduction (Johnson et al., 2004). Whether tadpoles develop deformities is a function of the timing and intensity of infections acquired during the early period of limb development (Johnson et al., 1999; Schotthoefer, Koehler et al., 2003). Identifying the conditions in aquatic habitats that influence the likelihood of transmission of *R. ondatrae* cercariae and tadpoles during this sensitive period is critical for understanding the emergence of this infectious disease.

The density of infective *R. ondatrae* cercariae in the environment is likely an important determinant of tadpole infection. Though the number of infected snails and the number of cercariae released by each infected snail will be central to determining the density of cercariae in the environment, factors that facilitate or hinder the survival or ability of cercariae to locate and infect tadpoles once released by snails will also be of critical significance. It has been proposed that cercariae are lost from the infective stage pool through predation by small aquatic predators (Anderson et al., 1978; Lafferty et al., 2006). Microcrustaceans and fish, for example, have been reported to eat cercariae of *Schistosoma mansoni* (Rowan, 1958; Knight et al., 1970; Christensen, 1979). However, few studies have demonstrated cercariophagic activity of predators. Therefore, it is important to begin to examine the possibility that predation represents a significant source of mortality that naturally limits cercaria transmission. In this study, we explored the cercariophagic potential of several invertebrate aquatic organisms, including members of the phyla Arthropoda (Odonata, Coleoptera, Anomopoda, Notostraca, Cyclopoida) and Cnidaria (Hydroida).

Preliminary observations of the potential cercariophagic activity were made with *Hydra* sp. (Hydroida), *Daphnia pulex* (Anomopoda), *Triops* sp. (Notostraca), copepods (Cyclopoida), damselfly larvae (Odonata,

TABLE I. Summary of the experimental trials conducted. Listed are the predator species tested, the numbers of *Ribeiroia ondatrae* cercariae placed into wells with each individual predator, the alternate food organisms offered (number of individuals) and whether or not cercariae were consumed.

	Preliminary observations		Feeding and preference trials		
Predator species	No. cercariae	Cercariae consumed?	No. cercariae/trial	No. predators/trial	Alternate prey species (No.)
Cnidaria					
Hydra sp.	10	+	10	14	*Daphnia pulex* (2)
Arthropoda					
Cyclopoid copepod	10	+	10	10	*Paramecium caudatum* (~23)
Daphnia pulex	10	−			
Triops sp.	10	−			
Dragonfly larvae	10	+	15	8	*Daphnia pulex* (5)
Damselfly larvae	10	+	15	8	*Daphnia pulex* (5)
Diving beetle	10	−			

Coenagrionidae), dragonfly larvae (Odonata, Libellulidae), and adult diving beetles (Coleoptera, Dytiscidae). Organisms for study were collected from the wild at ponds located in Champaign and Jasper Counties, Illinois, or supplied by Carolina Biological Supply (Burlington, North Carolina). All animals were maintained in the laboratory on a 12 hr dark:12 hr light cycle. Individuals (n = 2–3) of each invertebrate species were placed individually in wells of 6- or 24-well multiwell plates, depending on the size of the organisms, with carbon-filtered tap water and 10 *R. ondatrae* cercariae. Wells were observed under a dissecting microscope for a maximum of 20 min and the individual predators that consumed cercariae were recorded (Table I). All cercariae used in these initial observations and in our feeding trials were from naturally infected snails (*Planorbella trivolvis*) obtained from a privately owned pond in Urbana, Illinois (40°08′30″N, 88°11′30″W).

Separate feeding trials were conducted to quantify the rate at which *Hydra* sp., damselfly and dragonfly larvae, and cyclopoid copepods ate cercariae (Table I). Cercariae (0–5 hr old) were isolated from infected snails and placed in wells of multiwell plates. Individual predators (n = 8–14/trial) were introduced sequentially at 8–30-sec intervals into the wells with the cercariae (1 predator/well containing cercariae). The numbers of cercariae remaining in the wells at distinct time points following addition of the predators were recorded. Control wells (n = 2), in which cercariae but no predators were added, were established for each trial to determine the senescence rate of cercariae during the trial period. Different sized wells and volumes of water were used in the trials involving different predator species to adjust for the wide variation in the sizes of the predators (approximate lengths: <1 mm for copepods, 2–3 mm for *Hydra* sp., 7–9 mm odonate larvae). Thus, *Hydra* sp. and copepod trials were conducted in 15-mm-diameter wells of 24-well plates containing 160 μl and 2 ml of carbon-filtered tap water, respectively, and 10 *R. ondatrae* cercariae each. The number of cercariae remaining in wells was recorded at 5, 10, 30, and 60 min. Damselfly and dragonfly larvae trials were conducted in 35-mm-diameter wells of 6-well plates containing 8 ml of carbon-filtered tap water and 15 *R. ondatrae* cercariae. The number of cercariae remaining in wells was recorded at 2.5, 5, 7.5, and 10 min. A Sony DCR-TRV33 camcorder was used to capture video images of the wells during the feeding trials and assist with enumeration of the cercariae at the various time intervals, as the tape could be played back on a standard television monitor.

To determine if predators would consume cercariae when offered another prey item, preference feeding trials were conducted employing the same method as described, with wells in which cercariae and individuals of another prey item were added. The alternate prey item used for *Hydra* sp. and the damselfly and dragonfly larvae was *D. pulex*, and for the copepods it was *Paramecium caudatum* (Table I). *Daphnia pulex*, the copepods, and *P. caudatum* were reared in the laboratory with methods described in Dodson and Frey (2001) and Suarez et al. (1992). The *Hydra* sp. and copepod preference trials were conducted with different individuals than those used in the cercariae-only feeding trials. For the *Hydra* sp. preference trial, individual *Hydra* sp. (n = 14) were offered 2 *D. pulex* and 10 cercariae. For the copepod preference trial, individual copepods (n = 10) were offered the number of *P. caudatum* contained in 330 μl removed from a culture dish (average ± 1 SD number of *P. caudatum* individuals = 23.3 ± 8.12) and 10 cercariae. The same individual damselfly (n = 8) and dragonfly (n = 8) larvae used in the cercariae-only trials were used in the preference trials. In these trials, odonate larvae were offered 5 *D. pulex* individuals and 15 cercariae (Table I). The numbers of cercariae and alternate prey individuals offered to predators was arbitrarily chosen and adjusted based on the general sizes of the predators. The cercariae-only and preference trials were conducted within a 1-wk period. Prior to each feeding trial, predators fasted for 24 hr. At the end of the trials, predators were necropsied with the aid of a dissecting microscope 24 hr after cercariae consumption to assess them for the presence and number of metacercariae.

During the *Hydra* sp. feeding trial, some cercariae were consumed, and others were paralyzed by contact with the tentacles of *Hydra* sp. individuals. We considered these cercariae disabled and presumably noninfective, and therefore included them in our counts of cercariae killed by predators.

Numbers of cercariae eaten (or disabled) by *Hydra* sp. and the copepods were compared between cercariae only wells and cercariae plus alternate food wells using repeated measures analysis of variance. Paired *t*-tests were conducted at each time point to compare numbers of cercariae eaten with and without alternate food for the dragonfly and damselfly larvae trials. A level of $P < 0.05$ was chosen to detect significant differences (Zar, 1996).

Of the organisms tested, *R. ondatrae* cercariae were consumed by *Hydra* sp., the damselfly and dragonfly larvae, and the cyclopoid copepods (Table I). *Daphnia pulex* demonstrated no apparent response to, or interest, in the swimming cercariae. The diving beetles and *Triops* sp. also did not consume cercariae. When these organisms were placed in wells, they displayed erratic swimming behavior that seemed to distract them from eating anything. It is not clear if these organisms would consume cercariae under more natural conditions.

The dragonfly and damselfly larvae exhibited the greatest cercariophagic capability. Dragonfly larvae consumed, on average, about 7 (49.6%) of the cercariae offered in wells within 10 min, regardless of the presence of *D. pulex*. About half of the individuals tested consumed more than 50% of the cercariae offered during the trials and, on average, at least 1 cercaria was consumed within 2.5 min. The maximum number of cercariae consumed by any dragonfly during these 10-min trials was 14 (93.3% of cercariae offered). Although dragonflies also consumed *D. pulex* individuals (average, ± 1 SD in 10 min = 2.38, 1.303) during the preference trials, the presence of *D. pulex* did not affect the proportion of cercariae consumed by dragonflies at each time point (paired *t*-tests, all $P \geq 0.407$) (Fig. 1a). Damselflies also readily consumed cercariae. Most individuals ingested at least 1 cercariae within 2.5 min, and on average, 5 (33.7%) of the cercariae offered had been consumed within 10 min. Again, the presence of *D. pulex* had no effect on consumption of cercariae (paired *t*-tests, all $P \geq 0.761$), even though damselflies also ate *D. pulex* (average, 1 SD in 10 min = 3.13, 0.835) (Fig.

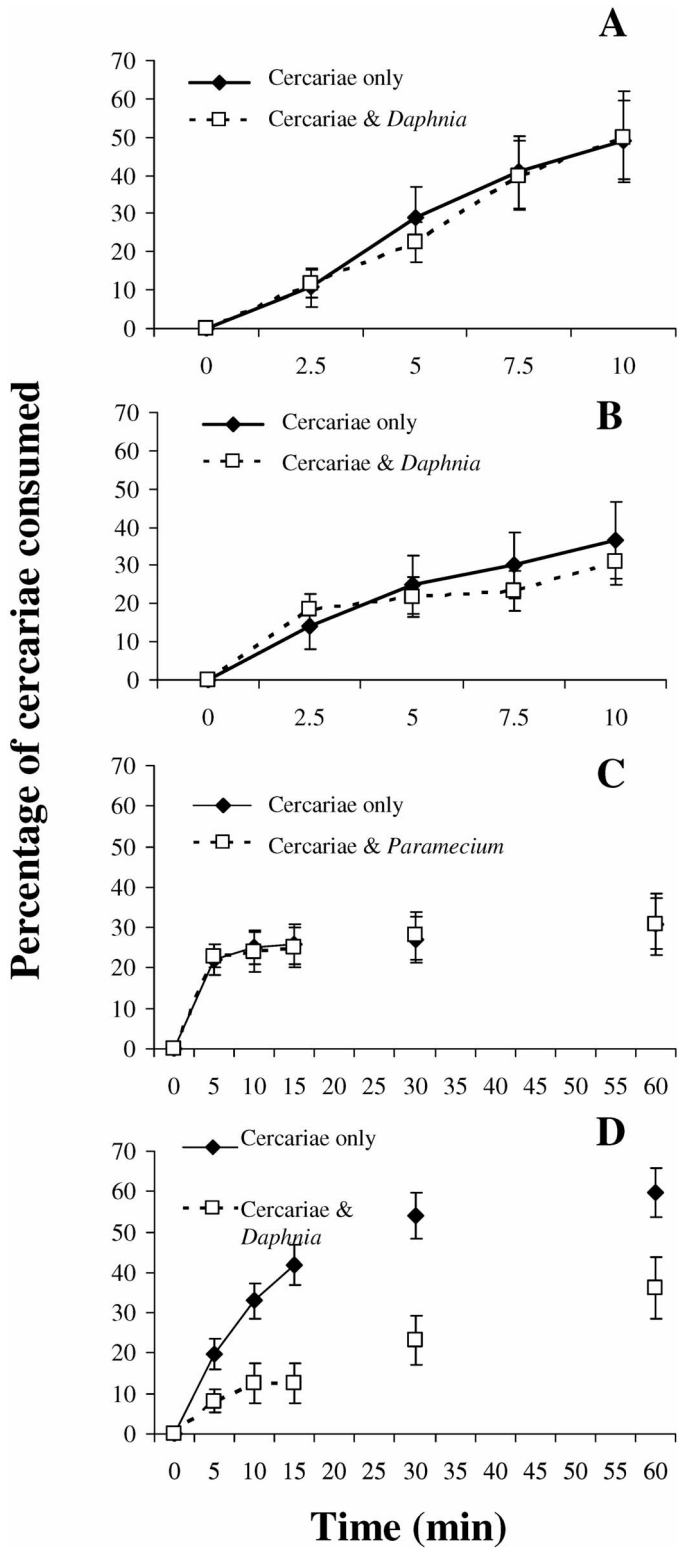

FIGURE 1. Percentages of *Ribeiroia ondatrae* cercariae consumed by (**A**) dragonfly larvae, (**B**) damselfly larvae, (**C**) cyclopoid copepods, and (**D**) *Hydra* sp. during feeding trials. Error bars show ±1 SE.

1b). Copepods and *Hydra* sp. consumed cercariae, but at slower rates than the odonate larvae. Copepods only ate about 2 cercariae in 10 min and, on average, 3 cercariae were ingested in 60 min. These numbers were unaffected by the presence of *P. caudatum* ($P = 0.982$), which were also eaten (Fig. 1c). The most cercariae consumed by any individual copepod was 6 in 60 min. Only 1–2 individuals consumed more than 50% of the cercariae offered during the feeding and preference trials. *Hydra* sp. disabled about 3 cercariae in 10 min and 6 cercariae in 60 min in the absence of the alternate food item, *D. pulex*. The presence of *D. pulex* significantly reduced the number of cercariae removed from wells by the *Hydra* sp. ($P = 0.003$), such that only 1 and 3 cercariae, on average, were disabled in 10 and 60 min, respectively (Fig. 1d). Ten of the 14 (71.4%) *Hydra* sp. individuals used in this feeding preference trial consumed 1 or both of the *D. pulex* offered to them with cercariae. None of the cercariae in any of the control wells died during the experiments. Necropsies conducted on predators 24 hr following trials revealed that none of the predators tested became infected with *R. ondatrae* metacercariae following consumption.

Transmission rates are of central importance to the dynamics of host–parasite populations (Anderson and May, 1978). Many factors may influence the transmission of cercariae between snails and their second intermediate hosts, including the densities, dispersion patterns, and demographics of the cercariae and host populations, as well as environmental factors, such as temperature (Evans et al., 1981; Evans, 1985; McCarthy, 1990; Pechenik and Fried, 1995; Schotthoefer, Cole, and Beasley, 2003; Ponder and Fried, 2004; Poteet, 2006). In addition, the composition of the aquatic community probably plays a role in at least 2 important ways. First, the availability of alternate hosts may interfere with transmission of cercariae to a particular host species by serving as competing targets for cercariae (e.g., McCarthy and Kanev, 1990). Second, cercariae may be preyed upon and killed by nonhost species, preventing transmission (Anderson et al., 1978; Lafferty et al., 2006).

Here we demonstrate that *R. ondatrae* cercariae are indeed consumed by invertebrate predators belonging to 3 major taxonomic groups and, for most of the predators examined, consumption was not altered by the availability of other prey items. In particular, odonate larvae displayed the highest consumption rate, and *Hydra* sp. and the cyclopoid copepods had the lowest rates of cercariophagia. Dragonfly and damselfly larvae are important predators in aquatic communities, especially in the benthic and littoral zones (Johnson, 1991). They are considered to be generalized carnivores, feeding on any prey item that is within their ability to capture and consume (Thompson, 1978; Westfall and Tennessen, 1996). Therefore, it is perhaps not surprising that they consumed the *R. ondatrae* cercariae. In contrast, copepods are considered omnivorous, but selective in their feeding, with some species tending to be more herbivorous than others (Williamson and Reid, 2001). For *Hydra* sp., handling time was obviously a factor in limiting the number of cercariae consumed during our trials. This was especially evident when cercariae were offered with *D. pulex*; the capture of an individual *D. pulex* would interfere with a *Hydra*'s ability to consume cercariae. Interestingly, however, the *Hydra*'s cercariacidal effects extended beyond their ingestion of cercariae and included killing by stinging.

Predation on cercariae by nonhost species has been described in the past. Rowan (1958) and Knight et al. (1970) reported on the cercariophagic activity of *Lebistes reticulatus* (guppy) on *Schistosoma mansoni* cercariae, and Christensen (1979) found that *D. pulex* and *Daphnia longispina* (Cladocera), plus *Notodromas monacha* and *Cypria ophthalmica* (Ostracoda), as well as *L. reticulates*, were predators of *S. mansoni* cercariae. Experiments conducted by Christensen (1979) and Christensen and Frandsen (1980) found that consumption of cercariae by predators interfered with transmission of *S. mansoni* cercariae to laboratory mice. The latter authors also demonstrated that the ability of predators to consume cercariae and reduce transmission to mice was dependent on the turbidity of the water used in experiments. In turbid water, predators did not interfere significantly with transmission, suggesting that predation upon cercariae was dependent on visual cues.

In our preliminary observations, we noted that when copepods were placed in wells containing water from their culture dishes, which contained a high algal content, they did not appear to feed on *R. ondatrae* cercariae as they did in our formal experiments, in which clear, carbon-filtered water was used. This suggests that algal content of water may affect cercariophagic behavior, perhaps by creating turbidity and decreasing visibility of cercariae, or by providing an alternate, stationary

food source that requires less effort to acquire. The effects of environmental factors, including water algal content, plant content, turbidity, and shade on cercariophagic activity, are worthy of further investigation. In addition to environmental factors, there are likely species-specific traits that may influence a predator's cercariophagic ability, i.e., foraging behavior, and microhabitat preferences, which we did not examine here, but which would provide important information on the predators most likely to influence cercariae transmission in nature. Moreover, the relative densities of potential predators versus the densities of cercariae and other prey items will probably also play a role in determining which predators are most important in different wetland settings. We arbitrarily chose the densities of 10 or 15 cercariae to feed to the predators in our trials, and conducted all trials in the evenings when cercariae were available from shedding snails. Obviously, the cercariophagic ability of different predators in nature may vary depending on the densities of cercariae available to them in their microhabitats during their active foraging times of the day. Further investigation of the cercariophagic activity of potential cercariae predators in the context of environmental and predatory–prey community dynamics will provide a framework by which to group likely predators and unlikely predators.

The observations reported here suggest the loss of *R. ondatrae* cercariae through predation may be an important regulatory function in transmission of cercariae to tadpoles in nature, and therefore may affect the occurrence of limb deformities in amphibian populations. We propose that there is need to examine determinants of cercariophagic activity by aquatic predators to assess the significance of such processes in protecting intermediate and definitive vertebrate hosts from trematode infections. The relevance of cercariophagic activity to the transmission dynamics of cercariae under natural versus anthropogenically altered conditions also needs to be explored.

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Phenotypic Variation in Infectivity of *Diplostomum spathaceum* Cercariae Within a Population

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ABSTRACT: The present study examined phenotypic variation in infectivity of *Diplostomum spathaceum* (Trematoda) cercariae within a natural population. Twelve infected *Lymnaea stagnalis* were collected from the field, and the infectivity of cercariae from individual snails was assessed under constant laboratory conditions. At a water temperature of 16.3 C, the mean infectivity of cercariae from the snails varied between 55.5% and 87.5%. Depending on the source of variation, this may have important ecological and evolutionary implications for both natural parasite populations and those occurring in aquaculture.

Variation in infectivity of parasites and susceptibility of their hosts and factors underlying this variation represent one of the fundamental features of the ecology of parasite-host relationships. Earlier, variation in transmission success of parasite infective stages was shown to be driven by environmental factors such as temperature (Lyholt and Buchmann, 1996; McCarthy, 1999), pollutants (Pietrock et al., 2002), parasite and host genetics (Lively, 1989; Koskela et al., 2002), and host ecological condition (Keas and Esch, 1997; Abrous et al., 2001). However, much of the research on the variation in parasite life history traits has focused on differences between geographical regions and laboratory lines (Wakelin and Goyal, 1996; Paterson and Viney, 2003). Studies examining variation within parasite populations are relatively scarce, although it may have important ecological and evolutionary implications. Variation, for example, may affect infection dynamics in individual hosts, and, when it is at least partly heritable (Lively, 1989; Carius et al., 2001), it forms the basis for evolutionary change. This is important also from an applied point of view when considering epidemiology and evolution of parasites and pathogens, which have established in intensive monocultures from wild populations. Thus, to evaluate these implications, we should first acquire estimates of life history variation within natural parasite populations, which include all the variation emerging from parasite and host characteristics.

In the present study, we examined within-population variation in the infectivity of *Diplostomum spathaceum* (Trematoda) cercariae by comparing cercariae released by different field-collected host individuals under standardized laboratory conditions. We were particularly interested in the level of variation in cercariae infectivity, but we also discuss possible causes and consequences of this variation.

Diplostomum spathaceum matures in the intestine of fish-eating birds, from which the eggs are released in the water with the bird's feces (Chappell et al., 1994). Eggs hatch to free-swimming miracidia, which infect the snail first intermediate host. Within the snail, the parasites develop into sporocysts that reproduce asexually, producing free-swimming cercariae. Thus, in the case of a single miracidium infection, cercariae produced by a snail are genetically identical. Cercariae infect the fish second intermediate host by penetrating the gills and skin and migrate to the eye lenses where they develop to metacercariae (Chappell et al., 1994). *Diplostomum spathaceum* is common in a range of freshwater fish species (Valtonen and Gibson, 1997) and is found also in farmed fish (Field and Irwin, 1994; Karvonen et al., 2006), where parasite-induced cataracts (Karvonen, Seppälä, and Valtonen, 2004) cause significant economic losses by impairing feeding of fish (Crowden and Broom, 1980; Owen et al., 1993) and increasing their susceptibility to predation (Seppälä et al., 2004, 2005). The parasite's life cycle is completed when an infected fish is eaten by a fish-eating bird (Chappell et al., 1994).

We collected a sample of *Lymnaea stagnalis* from Lake Huumonjärvi in northern Finland (65°06′N, 26°08′E) and brought the snails to the laboratory. We separated snails infected with *D. spathaceum* from other snails by observing the morphology and behavior of released cercariae in vivo (Niewiadomska, 1986). We placed 12 *D. spathaceum*–infected snails individually in glass jars containing 2 dL of water and allowed them to release cercariae for 4 hr. We estimated the number of cercariae released by taking 10 1-ml samples from each jar. We then exposed 10 randomly selected, 0+ yr-old rainbow trout (*Oncorhynchus mykiss*) to cercariae from each snail. Fish were obtained from a commercial fish farm where they had been reared in indoor tanks supplied with ground water. This ensured that fish had no previous experience with *D. spathaceum*. We conducted the exposures by placing the fish individually into containers with 0.5 L of water (16.3 C) and 70 cercariae for 20 min. We used standardized laboratory conditions to prevent possible effects of environmental factors on the cercariae. Thus, cercariae infectivity reflected the variation caused only by parasite and snail characteristics. After the exposure, we maintained the fish in 185-L tanks for 27 days. Although the parasite establishment in the eyes takes place very quickly, within 24 hr from exposure (Whyte et al., 1991), a longer maintenance period was used to allow parasite development and easy detection. Fish were maintained in large tanks because rainbow trout cannot be maintained in small containers for long periods. During the maintenance, water temperature decreased to 12.0 C, corresponding to ambient water temperature in nature during the study. We killed the fish with an overdose of 0.01% MS 222 (Sigma Chemical Co., St. Louis, Missouri), measured the length (±1 mm) and mass (±0.1 g) of each fish, and counted the number of parasites in lenses by dissecting the eyes. We used the proportion of parasites that had successfully established the eye lenses as an estimate of parasite infectivity. The relationship between the number of cercariae released by each snail, and their infectivity, was not examined because cercariae release by *D. spathaceum* is highly variable (Karvonen, Kirsi et al., 2004). Thus, the short time period (4 hr) used in the experiment was unlikely to provide reliable estimates of cercariae production.

Eighteen fish of the total of 120 exposed to cercariae died during the maintenance period (0–4 individuals from fish exposed to cercariae from each snail) and were excluded from further analysis because their parasite abundances could not be measured reliably. The average (±SE) body length and mass of the fish were 76 ± 1.0 mm and 5.1 ± 0.2 g, respectively, and fish exposed to cercariae from different snails did not differ in size (analysis of variance [ANOVA]: length: $F_{11} = 1.232$, $P = 0.278$; mass: $F_{11} = 1.216$, $P = 0.288$). Cercariae infectivity varied between different snail individuals (Kruskal-Wallis test: $\chi^2 = 28.749$, $P = 0.002$; Fig. 1) because the mean proportion of parasites successfully establishing the lenses ranged between 55.5% and 87.5%. The average infectivity of the cercariae released by individual snails did not affect the number of fish that died during the maintenance period (linear regression: $R^2 = 0.019$, $F_{1,10} = 0.195$, $P = 0.668$).

Corresponding to our earlier findings (Karvonen et al., 2005), we observed that *D. spathaceum* is highly infectious to naïve rainbow trout. However, the infectivity of cercariae released by individual snails was also highly variable, ranging between 55.5% and 87.5%. In general, variation in infectivity may be generated by parasite and host characteristics, as well as conditions of the surrounding environment. In this case, however, the experiment was conducted under constant laboratory conditions, which is why the effects of environmental factors such as temperature (Lyholt and Buchmann, 1996; McCarthy, 1999) directing on released cercariae are unlikely to explain the result. Thus, this study shows that when natural variation in both parasite and host characteristics is involved, life history variation within parasite populations can be high. Depending on the source of variation, this may have important ecological and evolutionary implications.

First, variation in parasite infectivity may be due to parasite charac-

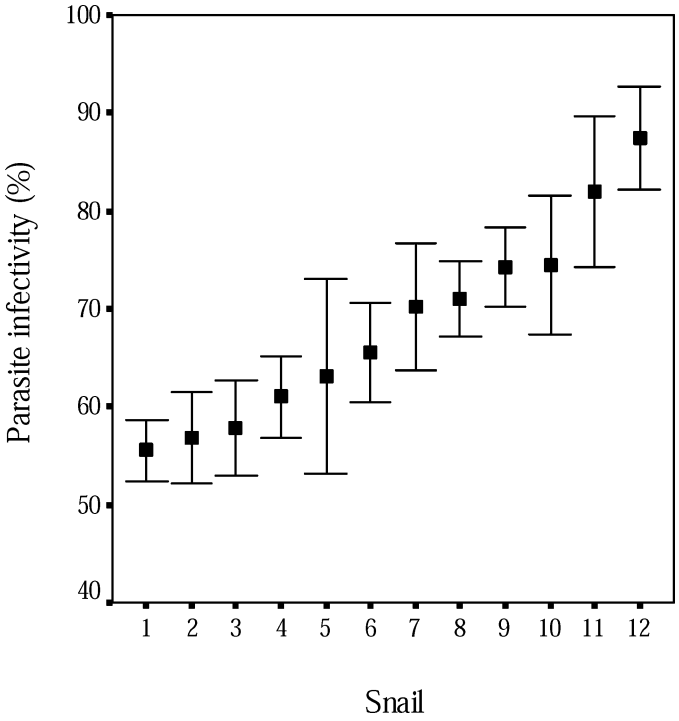

FIGURE 1. Average infectivity of *Diplostomum spathaceum* cercariae (±SE) produced by different snail individuals, in rank order. Infectivity is measured as the proportion of parasites successfully infecting fish eyes after exposure to 70 cercariae.

teristics such as genetic differences between cercariae produced by different snail individuals (see also Rauch et al., 2006). This is because snails become infected with different, sexually produced miracidia, and the subsequent asexual reproduction in the snails gives rise to clonal populations of cercariae. It must be stressed, however, that the snails of this study were collected from nature where it is possible that they have been infected with more than 1 miracidia (Rauch et al., 2005). However, in the case of multiple genotype infections, most of the cercariae are still produced by a single parasite genotype (Rauch et al., 2005), which suggests that the variation in infectivity may have a genetic basis. Furthermore, ecological factors such as the age of the sporocysts within a snail may affect cercariae characteristics. This could occur, for instance, if the amount of resources available for the parasite changes as the infection spreads within the host tissues. In this study, however, this is unlikely to explain the result because all infections had already advanced to a mature phase when most of the snail tissues were occupied by the parasite.

Second, in addition to parasite characteristics, those of the host may also affect infectivity of produced cercariae. For instance, it is possible that some of the individual snails show higher resistance to host exploitation by the parasite and thus reduce the quality (infectivity) of the cercariae. In this case both genetic and ecological factors may be involved. For example, stinging nettle (*Urtica dioica*) shows family-level variation in resistance against growth of a holoparasitic plant (*Cuscuta europaea*) (Koskela et al., 2002). On the other hand, in trematode-snail interactions, host individuals given low-quality diets release fewer cercariae compared to individuals fed high-quality diets (Keas and Esch, 1997; Sandland and Minchella, 2003). This suggests that host exploitation by parasites is dependent on host condition, which may also impact the quality of the cercariae released from the host.

Overall, variation in parasite infectivity, and factors underlying this variation, may influence parasite infection dynamics within host populations. If, for example, host exploitation by the parasites is reduced in poorly conditioned hosts, factors such as environmental stress may even reduce parasite population size (Pulkkinen and Ebert, 2004). Furthermore, if variation is at least partly heritable, it enables evolutionary change as a response to natural selection (Lively, 1989; Carius et al., 2001). This could have important economic consequences in aquaculture where *Diplostomum* spp. parasites commonly infect several fish species (Field and Irwin, 1994; Karvonen et al., 2006). Conditions at fish farms facilitate rapid completion of the life cycle because all host species required by the parasite are abundant in most cases. Thus, natural selection favoring most infective genotypes could lead rapidly to evolution of more infectious parasite strains (Gandon, 2004). Therefore, further studies examining those factors that lead to phenotypic variation in parasite infectivity are in high demand. The variation in cercariae infectivity between snails also raises an interesting methodological question regarding experimental designs using trematode cercariae. Because these experiments are usually conducted by extracting cercariae from infected hosts, it becomes especially important to have a sufficient replication of host individuals to gain comprehensive and reliable results of infection processes in these systems.

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